Differential Effects of Gram-positive and Gram-negative Inflammatory Stimuli on the Expression and Function of Energy Substrate Transporters in Human Mammary Epithelial cells

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ABSTRACT

Mastitis is often bacterial in origin. Lipoteichoic acid (LTA) and lipopolysaccharide (LPS), endotoxins from gram-positive and gram-negative bacteria, respectively, are potent inducers of mammary gland inflammation. Inflammation can alter expression of transporters responsible for transport of substrates important in synthesis of milk constituents and cellular metabolic energy. Since, gram-positive and gram-negative bacterial infections cause a different clinical course of mastitis, I investigated whether LTA and LPS differentially alter proton-coupled (MCT1) and sodium-coupled monocarboxylate transporter (SMCT1, SMCT2) expression and functional outcomes of altered expression.

Human mammary epithelial cells (MCF-12A) were incubated with 1 μg/mL LPS or LTA for 6, 12 and 24 hours and mRNA expression of TNF-α, IL-1β, IL-6, MCT1, SMCT1, and SMCT2 were measured using Quantitative RT-PCR. LPS decreased SMCT1, but increased SMCT2 expression after 6 h, while LTA increased MCT1 expression at 6 h, followed by gradual decrease in expression until 24 h. To know whether such differential changes in transporter expression by LPS and LTA could cause changes in cellular energy production, I quantified creatine (Cr) and high-energy phosphate substrates (CrP, ATP, ADP, AMP) and oxygen consumption rates using HPLC and Hansatech oxygen electrode, respectively. At 12 h, LPS increased concentrations of Cr, CrP, ATP and ADP, whereas LTA caused changes in CrP and ADP concentrations relative to control. Both LPS and LTA decreased oxygen consumption rates after 12 h. Furthermore, to know whether changes in transporter expression lead to differences in substrate availability, I performed uptake studies for carnitine using radiolabelled [^{3}H] L-carnitine. LPS and LTA challenge did not affect the affinity (K_m), but caused a 2-3-fold increase in maximal activity (V_{max}) of carnitine transport. Although increases in V_{max} were not significant, the increase in V_{max} after 12 h exposure by LPS and LTA corresponds to changes in mRNA expression of the OCTN2 transporter (previously reported in the laboratory).

In conclusion, LPS and LTA differentially alter mRNA expression of transporters, which leads to changes in cellular energy levels and oxygen consumption rates and possibly to changes in the functional activity of transporters. Whether such differences contribute to the different clinical course of mastitis warrants further investigation.

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I would like to dedicate this thesis to

My precious husband *Dr. Ramakrishna Gokaraju* for his endless encouragement and unconditional love

&

My Parents *M.S.V.P. Raju*, *M.V. Subbamma*, and my brothers *Anand*, *Nagaraju* who are always special to me

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
Ci	Curie
CLRs	C-type lectin receptors
Cr	Creatine
CrP	Creatine phosphate
C_T	Crossing point
DAMPs	Danger associated molecular patterns
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
FABP	Fatty acid binding proteins
FATP	Fatty acid transport proteins
GLUT	Glucose transporters
$HClO_4$	Perchloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HQC	High quality control
IFN-γ	Interferon-γ
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
IRF-3	Interferon regulatory factor-3
K_2HPO_4	Dipotassium hydrogen phosphate
KCl	Potassium chloride

KH_2PO_4	Potassium dihydrogen phosphate
K_m	Michaelis constant
KOH	Potassium hydroxide
LPS	Lipopolysacccharide
LQC	Low quality control
LTA	Lipoteichoic acid
MCF-12A	Human mammary epithelial cell line
MCT	Monocarboxylate transporter
$MgSO_4$	Magnesium chloride
mL	Milliliter
mM	Millimolar
MQC	Mid quality control
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response
Na_2HPO_4	Disodium phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NF-κB	Nuclear transcription factor κB
NLRs	Nucleotide binding oligomerization domain like receptors
OCR	Oxygen consumption rate
OCTN	Organic cation / carnitine transporter
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffer saline
PRRs	Pattern recognition receptors
RLRs	Retinoic acid-inducible gene (RIG)-I-like receptors
rpm	Revolutions per minute
RT-PCR	Reverse transcription-polymerase chain reaction
SARAM	Sterile-alpha and Armadillo motif-containing protein
SCFA	Short chain fatty acids
SGLT	Sodium dependent secondary active Na^+/glucose transporter
SLC	Solute carrier transporter

SMCT	Sodium-coupled monocarboxylate transporter
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptors
T_m	Annealing temperature
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing interferon beta
V_{max}	Maximal binding capacity
μM	Micromolar

1. INTRODUCTION

Mastitis, an inflammation of breast tissue, occurs in both lactating and non-lactating women. Usually it is more common in the lactating mammary gland. Mastitis occurs due to pathogens such as bacteria, viruses, and yeast. However, the vast majority of infectious mastitis is due to bacterial origin. Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacteria are the common sources of bacterial infections in the lactating mammary gland. Infections induced by *E.coli* are acute and cause more severe damage to the mammary gland, whereas infections due to *S.aureus* usually disappear within a few days and tend to cause a low-grade chronic infection.

Much research has explored the role of immunological factors in mastitis. Gram positive and Gram negative bacteria elicit different innate immune responses during mastitis in bovine mammary epithelial cells. Differences in stimulation of the innate immune response, neutrophil and macrophage recruitment, and other immunological factors play critical roles in the clinical course of gram-negative and gram-positive mastitis. However, stimulation of the innate immune response may result in other changes in the mammary epithelium that could affect mammary epithelial cell function and, in turn, the clinical course of mastitis. Investigations on different epithelial barriers have shown that chronic inflammation may lead to or be the result of deficient cellular energy metabolism either as a result of decreased availability or utilization of cellular energy substrates.

Transporters play an important role in making nutrients available to mammary epithelial cells for cellular metabolic energy production and synthesis of milk constituents. In mammals, glucose, fatty acid and L-carnitine transporters play a significant role in making energy substrates available to epithelial cells to produce ATP. Monocarboxylate transporters contribute to the uptake of intermediate metabolites formed in the metabolism of glucose and fatty acids. Changes in such transporter expression levels during mammary gland inflammation may affect the availability of energy substrates and nutrients, their utilization, and the biosynthetic capacity of the mammary epithelium. Since gram negative and gram positive bacterial infections result in a different clinical course of mastitis, differential changes in the transporter expression profiles may contribute to the different clinical outcomes of mastitis. Therefore, in my thesis I evaluate differences in pro-inflammatory cytokine release following gram positive and gram negative

bacterial stimulation, and the differential effect of the stimulation of the innate immune response on mammary epithelial cellular function and energy metabolism and energy substrate transporter expression.

2. LITERATURE REVIEW

2.1. Mastitis

Mastitis is broadly defined as an inflammation of the mammary gland, including intramammary tissues and anatomical structures such as milk ducts and nipples. The mammary gland is the sole nutrient providing organ to nursing offspring and provides a vital survival and evolutionary advantage to mammalian species. Inflammation of the mammary gland can occur in both lactating and non-lactating mammals, but is more common during lactation, particularly the early stage of lactation (1, 2). In human mastitis, 74-95% of cases are observed within first 3 months (2, 3). Mastitis can be unilateral or bilateral and, in the latter case in humans, one of the breasts is usually more affected than the other. Mastitis can cause early weaning (2) and, hence, is an important health concern in women in developing countries. According to the World Health Organization (2000), the occurrence of mastitis varies between 2% to 33% and the global incidence of mastitis is under 10% in lactating women (4).

Mastitis disrupts mammary gland function, which leads to both physiologic and metabolic changes in the mammary gland. It can cause physical damage to the mammary epithelium, the basic milk production unit of the mammary gland, which leads to changes in quantity and quality of breast milk by enhancing permeability of solutes across the mammary epithelial cells (5). For example, mastitis can decrease high quality milk protein casein and increase low quality whey protein levels in the milk. Furthermore, it can increase the passage of serum albumin, immunoglobulins, sodium, and chloride into milk by increasing the permeability in mammary epithelial cells (6). Inflammation of the mammary gland can also cause changes in the metabolic activity of mammary epithelial cells, which, in turn, results in changes in the composition of the milk constituents. For instance, the concentration of lactose in mastitic milk decreases due to impaired acidification in mammary epithelial cells (7). Furthermore, mastitis can increase the production of enzymes that decompose milk proteins. For example, proteolysis activity of proteinase enzymes in milk increases by more than two fold during mastitis and causes breakdown of milk protein, casein (6). Hence, mastitis can cause significant changes in the composition and volume of milk by causing changes in physiologic and metabolic activity of the mammary epithelium.

2.1.1. Classification of Mastitis

Depending on the criteria used, mastitis can be classified into several subgroups such as lactational and non-lactational mastitis (lactation stage), clinical and subclinical mastitis (clinical manifestations), and infectious and non-infectious mastitis (course) (Figure-2.1.).

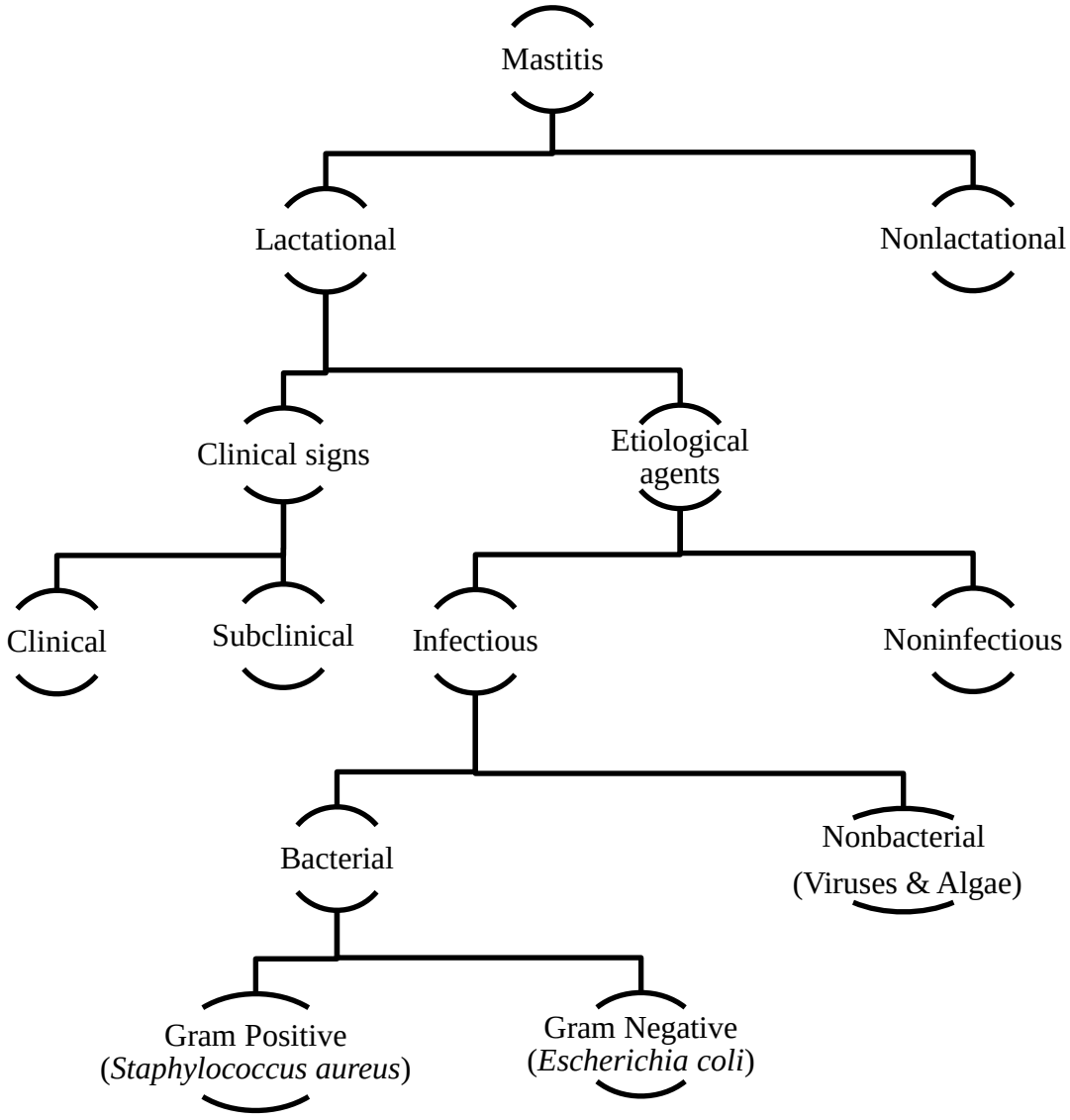

Figure 2.1. Classification of mastitis in mammals depending on lactation stage, clinical signs, and etiological agents (Adapted from G. Andres Contreras (1)).

Lactational mastitis is characterized by an acute inflammation of the interlobular connective tissue in the mammary gland during lactation stage (8). Non-lactational mastitis is

similar to lactational mastitis, but occurs in non-lactating women. It mainly comprises periductal mastitis and breast abscesses (9). Clinical mastitis is characterized by presence of external clinical signs including pain, hyperemia, increase in mammary gland size and density, and fever (10). In contrast, subclinical mastitis is characterized by the presence of internal changes in the mammary gland such as decrease in milk secretion, high bacterial counts in milk, and an increase in the milk sodium/potassium ratio (Na^+/K^+) (11). Infectious and non-infectious mastitis directly relates the clinical course of mastitis to the clinical manifestations and the causative pathogen (1).

2.1.2. Infectious Causes of Mastitis

Mastitis can be caused by a number of infectious pathogens such as bacteria, viruses and yeast. However, the vast majority of mastitis is due to bacterial origin. Gram-positive (*Staphylococcus aureus, Staphylococcus albus, Streptococcus uberis and Streptococcus agalactiae*) and Gram-negative (*Escherichia coli*) bacteria are the common sources of bacterial infections in the mammary gland (1).

2.1.2.1. Gram-positive and Gram-negative bacteria

Bacteria can be divided into two different groups depending on their staining characteristics by Gram-method, namely Gram-positive and Gram-negative bacteria (12). These bacteria differ in many ways. First, both bacteria consist of a cytoplasmic membrane, surrounded by a characteristic structure known as peptidoglycan layer (PG). The PG layer contributes a major portion of cell wall in gram-positive bacteria, whereas, it is only a thin layer in gram-negative bacteria (Figure 2.2.) (12). Furthermore, when both bacterial groups are stained by the Gram-method, gram-positive bacteria stain in violet due to the presence of a greater peptidoglycan layer in their cell wall, while gram-negative bacteria stain in pink, which suggests the presence of a thin layer of peptidoglycan layer. Second, gram-negative bacteria have an outer membrane surrounding the PG layer. It consists of phospholipids and lipopolysaccharide (LPS). LPS is a potent stimulant of the innate immune system among all other components of gram-negative bacteria. Gram-positive bacteria do not have the outer membrane surrounding the PG layer and the matrix of peptidoglycan layer consists of lipoteichoic acids (LTA) (12).

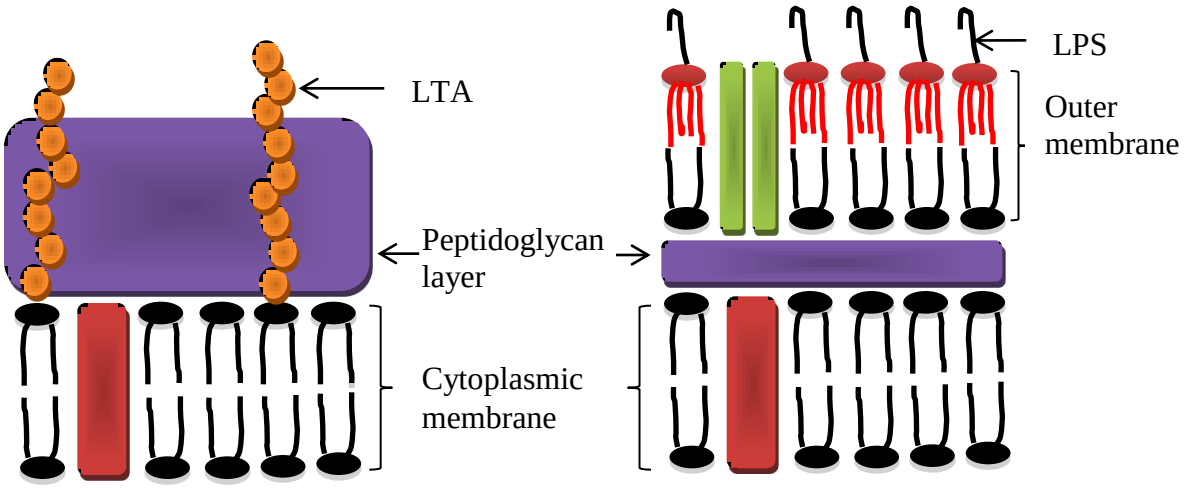

Figure 2.2 Cell wall structures of (a) Gram-positive and (b) Gram-negative bacteria (Adapted from Shizuko Akira (12)).

2.1.3. Gram-positive and Gram-negative bacteria elicit a different clinical course of mastitis

Due to the differences in the cell wall constituents of Gram-positive and Gram-negative bacteria, these pathogens may stimulate different receptors of the innate immune cells and mammary epithelial cells and thereby can result in different clinical course of mammary gland infections (13). Hence, infections induced by Gram-negative bacteria are usually acute and cause more severe damage to the mammary gland. Clinical manifestations of infections due to Gram-positive bacteria usually disappear within a few days, but persist as a low-grade chronic infection (13, 14).

Much research has explored the role of immunological factors in mastitis. Gram positive and gram negative bacteria elicit different innate immune responses during mastitis and investigations on bovine mammary epithelial cells indicate temporal differences in the changes in mRNA expression and release of cytokines (IL-1 β and TNF-α) during LPS- and LTA-induced inflammatory stimulation (13). Differences in stimulation of the innate immune response, neutrophil and macrophage recruitment and other immunological factors play critical roles in the clinical course of gram-negative and gram-positive mastitis. For instance, inoculation of mammary epithelial cells with *E.coli* bacteria increases the complement system C5a concentrations along with influx of neutrophils, whereas, C5a is usually not detected in cells

inoculated with *S. aureus* bacteria (15). Similarly, an iron binding glycoprotein, lactoferrin, binds to the Lipid A of gram-negative bacterial lipopolysaccharides (LPS). Thus, lactoferrin neutralizes LPS induced inflammation by preventing its interaction with LPS-binding protein (LBP) and membrane and soluble CD14, while binding of gram-positive bacteria *S. agalactiae* to lactoferrin activates the classical complement pathway resulting in opsonization of bacteria by lysozyme enzymes (16, 17). Stimulation of the innate immune response may result in other changes in the mammary epithelial cells that could affect mammary epithelial cell function and, in turn, the clinical course of mastitis. Investigations on different epithelial barriers have shown that chronic inflammation may lead to or be the result of deficient cellular energy metabolism either as a result of decreased availability or utilization of cellular energy substrates (e.g. carbohydrates and lipids, the major substrates for cellular energy) (18).

2.2. Immune system in mammals

The immune system in mammals is separated into two distinct categories: innate immunity and acquired immunity (12). Innate immunity, also known as nonspecific responsiveness, provides the first line of defense against invading microorganisms or pathogens. The nonspecific responses are present during normal, healthy conditions, and are quickly activated upon exposure to numerous pathogenic stimuli. These responses are not augmented by repeated exposure to the same pathogen. The defense mechanism of innate immunity system is mediated by professional immune cells, such as macrophages, dendritic cells, neutrophils, and by nonprofessional immune cells, such as epithelial cells and endothelial cells (19). Adequate functioning of the innate immune system eliminates most of the pathogens within a short period of time and before the activation of acquired immune system.

When pathogens are not completely eliminated by the innate immune system, the acquired or specific immune system becomes activated. The acquired immune system provides an addition to the overall immune strategy by eliminating pathogens in the later phase of infection, i.e. it provides a second-line of defense against re-exposure to the same pathogen (19). Recognition of pathogens by acquired immune system depends on the generation of random and highly diverse repertoire of antigen receptors - T-cell receptors and B-cell receptors, followed by clonal selection and expansion of receptors with relevant specificities (20). Although B-cells are able to recognize free antigens, T-cells can only recognize specific antigens presented to them through genetically diverse, membrane bound proteins known as major histocompatibility

complex (MHC) molecules. These mechanisms account for the generation of the immunological memory to a specific antigen, which provides considerably stronger, long lasting and often more effective response in clearing the pathogen. However, the acquired immune response has two main limitations (20). One, randomly generated antigen receptors cannot recognize specific antigens by themselves. Second, they require certain immunoglobulin genes to generate specific clones and differentiate into effector cells before they contribute to defense against specific pathogen. As a consequence, the specific immune responses are delayed for few days, which can cause more damage in the host due to increase in microbial invaders (20). Furthermore, the specific immune system does not function independently. Almost every aspect of specific immune response is controlled by instructive signals elicited from the innate immune system. Hence, the innate immune system is crucial in recognizing pathogens, in providing first-line defense mechanisms, and in activating acquired immune responses against pathogens as soon as they enter into the body. The innate immunity of the human mammary gland is mediated by immune cells such as neutrophils and macrophages, as well as by mammary epithelial cells (19, 20).

2.2.1. Role of the immune cells in innate immunity

The mammary gland consists of resident phagocytic immune cells such as macrophages and the migrating immune cells such as dendritic cells and neutrophils. In general, macrophages are the predominant cell type present in healthy mammary gland. The primary function of the macrophages is phagocytosis, intracellular killing of invading microorganisms and removal of milk fat from involuting glands (21). Macrophages act as initiators of inflammation. Upon stimulation of macrophages by phagocytosis of invading pathogen, they release chemotactic activity to recruit neutrophils to the site of infection (22). This neutrophil recruitment from the circulation to the site of infection is essential in providing defense to the mammary gland against invading pathogens. The promptitude of recruitment and the amount of recruited neutrophils determine the outcome of infection (23). For example, stimulation of macrophages by LPS induces an acute phase response to eliminate the bacteria. In such a case, neutrophil recruitment ceases in response to inflammatory stimuli and returns to healthy levels. This is what is observed in acute clinical mastitis, mainly by *E. coli*, and accompanied by a spontaneous cure of the infected mammary gland within a few days after the beginning of the infection (24). Certain bacteria are able to survive this immediate host response, and the inflammation and recruitment

of leukocytes continue. This prolonged diapedesis of leukocytes causes damage to mammary gland tissue, resulting in chronic and subclinical type of infections (25). For example, mastitis due to *S. aureus* continues for several months after the beginning of the infection and the infected gland contains neutrophils as the major cells in it. Hence, the activities of resident and migrating immune cells during early stages of pathogenesis play a pivotal role in the establishment of intramammary infection (22, 23).

2.2.2. Role of epithelial cells in innate immunity

During lactation, the mammary epithelium becomes a polarized epithelium where tight junctions between epithelial cells result in the formation of a mechanical barrier between the lumen of the mammary gland and the mammary gland tissue (26, 27). There is a belief that "good fences make good neighbors", likewise, the lactating mammary epithelium plays a crucial role in innate immunity by providing a 'good fence' (first line of defense) against microbial entry (28). The mammary epithelium also plays a key role in providing innate defense against pathogens either by directly killing them or by signaling to release inflammatory mediators such as cytokines and chemokines, which helps to recruit more leucocytes to the site of microbial entry and directly kills the pathogens (28-30). Microorganisms that enter into the human body must cross the mammary epithelial barrier to cause an infection in the mammary gland. Epithelial cells are the first cells that come in contact with pathogens when they enter into the body (30, 31). All these defense mechanisms of innate immune system are highly active only upon sensing the pathogens or microbial peptides through specific receptors present in them. These receptors are known as pattern recognition receptors.

2.2.2.1. Pattern recognition receptors

Epithelial cells and immune cells are known to express germ line-encoded pattern-recognition receptors. Several types of PRRs have been identified to recognize the pathogens in immune cells and epithelial cells (12, 31). All these PRRs possess some common characteristics (12): First, PRRs recognize the molecular patterns that are broadly shared by pathogens such as bacteria, viruses, protozoans, fungi and double stranded RNA. Such highly conserved molecular patterns are known as pathogen associated molecular patterns (PAMPs). PAMPs of microorganisms are associated either with the external surface of pathogens or their internal elements, crucial to their metabolic process and therefore essential for the survival of microorganisms. Second, PRRs detect microorganisms regardless of their life cycle stage. Third,

PRRs are nonclonal, germline encoded and expressed on all host cells of a given type, and independent of immunological memory. Last, PRRs recognize the host molecules released from the injured cells that are exposed to pathogens and their toxins. These molecules include tumor necrosis factor (TNF-α), interleukins (IL-1β), host derived DNA, and/or RNA are known as danger signals or damage associated molecular patterns (DAMPs). Recognition of PAMPs and DAMPs by different PRRs activates intracellular signaling pathways resulting in release of distinct effector molecules such as pro-inflammatory cytokines that are involved in defense mechanisms against pathogens, inflammation, and in modulating adaptive immunity (31).

PRRs are classified into four different families: Membrane bound Toll like receptors (TLRs) and C-type lectin receptors (CLRs), cytoplasmic Nucleotide binging oligomerization domain like receptors (NLRs) and Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) (31). Of all these families, TLR is one of the best-known families of PRRs responsible for sensing pathogens from extracellular surfaces and intracellular vesicles. Hence, in the following sections I concentrate on innate immune recognition of bacteria by TLRs and their intracellular signaling pathways for the release of pro-inflammatory cytokines and their effects on cellular metabolism.

2.2.2.2. Toll-like receptors

TLRs are highly conserved class I transmembrane proteins, which consist of an extracellular N-terminal leucine rich repeats responsible for recognition of pathogens, and a transmembrane domain followed by cytoplasmic Toll/interleukin-1 receptor (TIR) domains required for initiation of intracellular signaling (32). Upon ligand binding, TLRs undergo conformational changes required for recruitment of TIR domain-containing adaptors molecules. Five TIR domain-containing adaptor molecules have been identified to play an important role in TLR signaling pathways, namely myeloid differentiation primary response (MyD88), TIR domain-containing adaptor inducing interferon beta (TRICAM-1 or TRIF), TRIF-related adaptor molecule (TRAM), TIR domain-containing adaptor protein (TIRAP), and Sterile-alpha and Armadillo motif-containing protein (SARAM) (12, 33). Different TLRs use different combinations of adaptor proteins to determine downstream signaling. TLR signaling pathways can be classified into two distinct pathways depending on the usage of distinct adaptor molecules, MyD88 and TRIF (32, 33). Activation of MyD88-dependent pathways is responsible for activation of transcription factors NF-κB, which drives the induction of genes encoding

inflammatory cytokines, chemokines and antimicrobial factors (31-33). Similarly, TRIF pathways activate transcription factors NF-κB and Interferon regulatory factor-3 (IRF-3) responsible for induction of inflammatory cytokines and type-I interferons respectively (Figure-2.3.) (34). In this way, TLRs recognize molecular structures derived from bacteria, viruses, fungi, or parasites. To date, 10 members of TLRs have been identified in humans. Depending on their cellular localization of Toll-like receptors, they are classified in to two groups (35). Plasma membrane localized TLRs, TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, recognize microbial membrane components such as proteins, lipoproteins and lipids; whereas endosomal TLRs, TLR3, TLR7, TLR8, and TLR9, recognize microbial nucleic acids. TLR4 recognizes the LPS of Gram-negative bacteria and TLR2 either alone or along with TLR1 or TLR6 recognizes peptidoglycan, lipoprotein and lipoteichoic acid of Gram-positive bacteria (12, 31, 34).

2.2.2.2.1. Recognition of gram-negative bacteria by TLR4

In mammals, TLR4 is the signal transducing receptor activated by bacterial LPS. LPS, also known as endotoxin, is the most potent immunostimulant among all the cell wall components of Gram-negative bacteria. A lipid portion of LPS, lipid A, is responsible for most of the pathogenic phenomena associated with Gram-negative bacterial infections. When gram negative bacteria enter into the body, the bacterial LPS associates with LPS binding protein (LBP) present in the blood stream, and then binds to a glycosylphosphatidylinositol linked protein, CD14, which then transfers them to TLR4. TLR4 homodimerizes and forms a complex with the extracellular protein MD2. LPS is then transferred to MD2, which associates with extracellular portion of TLR4. Thus, cells need both MD2 and TLR4 in order to recognize LPS (12, 32). Activation of TLR4 initially recruits TIRAP at the plasma membrane and subsequently facilitates the recruitment of MyD88 to trigger the initial activation of transcription factors NF-κB. TLR4 subsequently undergoes dynamin-dependent endocytosis and is trafficked to the endosome, where it forms a signaling complex with TRAM and TRIF to initiate the TRIF dependent pathway. Activation of the TRIF dependent pathway leads to IRF-3 activation as well as late phase activation of NF-κB. Thus, TLR4 initially activatesthe MyD88-dependent pathway followed by activation of the TRIF-dependent pathway. Activation of both these pathways is necessary for the induction of inflammatory cytokines by TLR4 receptor (31, 34, 35).

2.2.2.2.2. Recognition of gram-positive bacteria by TLR2

TLR2 plays a major role in the detection of Lipotechoic acid (LTA) of Gram-positive bacteria. However, it also recognizes lipoproteins and peptidoglycans present in both Gram positive and Gram negative bacteria (31). TLR2 forms heterodimers with TLR1 or TLR6, which appear to be involved in the discrimination of subtle changes in the lipid portion of lipoproteins. Specifically, TLR 1/2 heterodimer recognizes triacylated lipopeptides and TLR1/6 heterodimer recognizes diacylated lipopeptides from gram-positive bacteria (31). The importance of TLR2 against Gram-positive bacteria has been studied using TLR2-deficient (TLR2-/-) mice. These studies found that the TLR2 deficient mice were highly susceptible to challenge with *Staphylococcus aureus* (36, 37). A polymorphism in the human TLR2 gene (Arg753Gln) has been shown to be associated with a reduced response to different bacterial lipoproteins (38). Activation of TLR2, TLR1/2, and TLR2/6 cause induction in inflammatory cytokines through an MyD88-dependent pathway (33, 35).

2.2.2.3. Release of cytokines due to LTA and LPS induced inflammation

Cytokines are small, nonstructural proteins produced in response to infection and inflammation by immune cells. Cytokines play different roles during infection or inflammation such as differentiation, activation and maturation of various immune cells. (39). During normal conditions, the cytokines are usually expressed at low static levels in the circulation, but concentrations are dramatically elevated during certain disease conditions. Depending on disease conditions/specific local microenvironments these cytokines exert either pro-inflammatory or anti-inflammatory effects, or both.

Activation of the MyD88-pathway by TLR2 and TLR4 upon recognition of LTA and LPS, respectively, activates transcription factors NF-κB and MAPKs to release pro-inflammatory cytokines such as IL-1β, and TNF-α. However, LPS also activates the TRIF dependent pathway, which further activates IRF-3 leading to the transcription of genes encoding for IFN-γ (32, 35). IFN-γ possesses both antiviral and antibacterial activity. IFN-γ is also considered as a pro-inflammatory cytokine because it increases the activity of TNF-α (39).

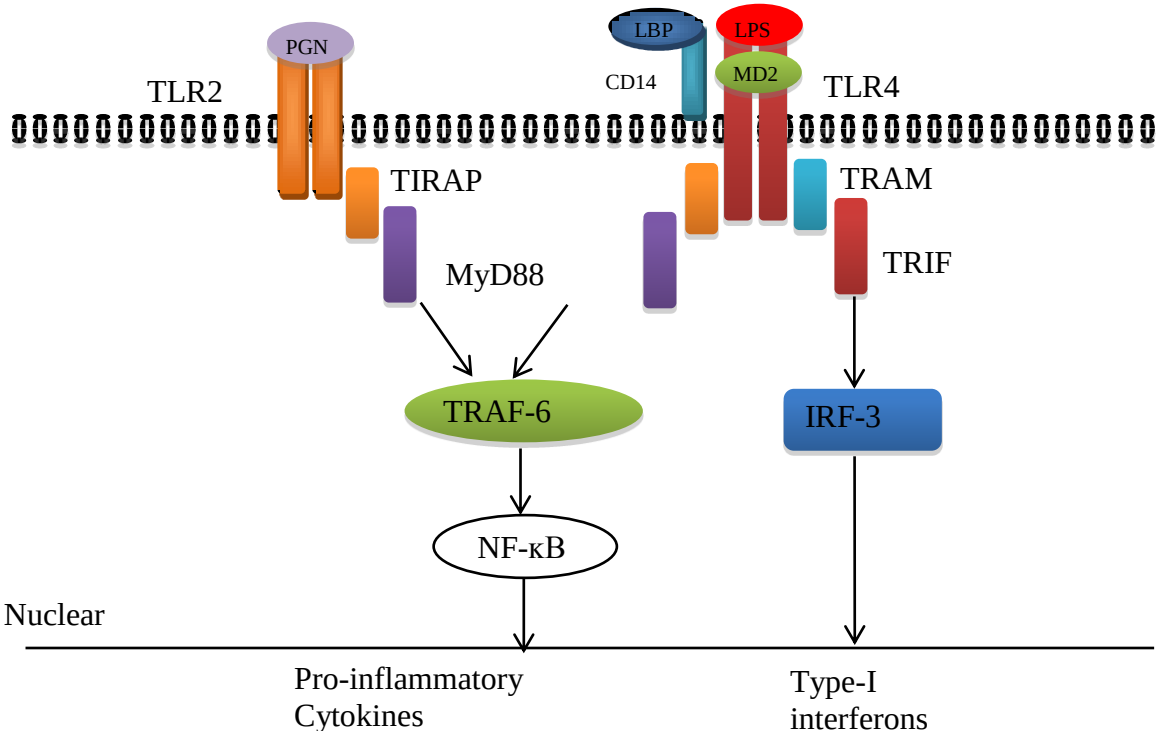

Figure 2.3. Overview of LTA/TLR2 and LPS/TLR4 signaling in mammary epithelial cells. Recognition of peptidoglycans (PGN) of gram positive bacteria is mediated by TLR2 receptor and recognition of LPS is facilitated by LBP, CD14 and is mediated by TLR4/MD-2 receptor co complex. PGN and LPS induce genes related to pro-inflammatory cytokines through MyD88 dependent pathway. However, LPS also release type-I interferons through TRIF dependent path way (Adapted from Yong-Chen Lu et.al. (34)).

The release of pro-inflammatory cytokines following LPS and LTA stimulation can have local and systemic effects. At the systemic level, they mediate acute phase reactions such as fever and release of acute phase proteins (40). For example, the LPS of *E.coli* is often associated with acute symptoms at the systemic level by elevating TNF-α levels in the serum and milk. TNF-α causes endotoxin shock and elevated concentrations were found in cows that had died due to acute *E.coli* mastitis during the periparturient period (41). At the local level, they primarily act in an autocrine and paracrine manner. For example, the intestinal epithelium of humans and rats is reported to make IL-6 cytokine during inflammation, and also express IL-6 receptors (42, 43). Primary cultures of mouse hepatocytes and liver cells of many mammalian species have shown the presence of IL-1α transcripts and show response to IL-1 with changes in acute phase protein production (44). Cytokines are extremely potent at low concentrations. They

exert their effect by binding to specific receptors on immune cells and epithelial cells. For instance, IL-1 exerts its effect by binding to IL-1 receptors (IL-1RI and IL-1RII) and IL-1R accessory proteins (IL-1R-AcP) (45). TNF-α exerts its effect by binding to two receptors, p55 and p75 receptors (46). They exert their potency through different cascades of pathways and networks, where one cytokine leads to the secretion of other cytokines, which often results in amplification or synergistic effects. TNF-α and IL-1 together exhibit synergistic enhancement in IL-6 production in human thymic epithelium and in rat intestinal epithelial cells (47, 48). However, some cytokines have reciprocal effects to other cytokines, which results in negative feedback loops. For example, release of anti-inflammatory cytokines such as IL-4, IL-10, and transforming growth factor (TGF-β) suppress the production of pro-inflammatory cytokines IL-1, TNF-α and chemokines such as IL-8 (39). Furthermore, the exaggerated effects of cytokines in acute and chronic inflammatory conditions may be due to unregulated production of pro-inflammatory cytokines or due to inadequate production of anti-inflammatory cytokines (49).

2.3. Mammary epithelium

The mammary epithelium is formed by a single layer of mammary epithelial cells. During lactation, mammary epithelial cells undergo a number of changes such as formation of tight junctions between the adjacent epithelial cells. The polarity of the epithelial cells is maintained by these tight junctions to create a distinct outward-facing apical domain and a distinct inward-facing basolateral domain (26, 28). As expected for highly active secretory cells, the cytoplasm of lactating epithelial cells consists of numerous mitochondria, extensive rough endoplasmic reticulum, and well-developed Golgi apparatus to generate more cellular energy required for the synthesis of milk constituents and its transport across the mammary epithelial cells (50).

2.3.1. Composition of milk

Maternal milk is a complex biological fluid composed of many different constituents. These include proteins, carbohydrates, fat-globule membranes, vitamins, minerals and non-nutrient bioactive compounds such as enzymes, hormones, epithelial cells and immune cells (50). The critical milk constituents are categorized into two groups, macronutrients and micronutrients, based on their physical and physiological properties.

Macronutrients are nutrients needed in large amounts. These include lipids, proteins and carbohydrates (51). Macronutrients either collectively or independently provide the metabolic

energy required to maintain all metabolic functions in a living organism (52). Lipids constitute 3-5% of human breast milk. The majority of the milk lipids are derived from maternal blood circulation and only a small portion of lipids are synthesized *de novo* by the mammary epithelial cells (53, 54). Hence, during lactation, dietary lipid intake can have profound effect on milk lipid composition (55). Milk proteins are also important macronutrients and constitute 0.9% of human milk. Milk proteins are mainly synthesized by mammary epithelial cells and a minor portion is obtained from maternal circulation. They provide essential amino acids required for neonatal growth and development (56, 57). Another major constituent is lactose, a carbohydrate which constitutes 5-7% of milk. Mammary epithelial cells synthesize the major portion of milk lactose but some lactose is derived from the maternal circulation (58). Depending on the stage of lactation, the composition of these macronutrients varies. Any drastic disturbance in the balance of these macronutrients in milk has detrimental outcomes to the nursing neonate (59). For example, an association between mothers exposed to a high carbohydrate diet during the lactation and chronic hyperinsulinemia and adult-onset diabetes of the offspring suggests a permanent programming of β-cell function in the nursing offspring (60).

Micronutrients are nutrients required in small amounts in breast milk. These include vitamins, minerals such as calcium and magnesium, trace minerals such as iron, copper and zinc, and smaller peptides (61). These micronutrients contribute to a number of biochemical pathways such as cell signaling pathways, hormones and growth factors. Imbalance or deficiency of micronutrients has detrimental effects on the developing offspring (62). For example, minor iron deficiencies in milk altered the expression of major milk proteins, β-casein and the whey-acidic protein (WAP) (63).

2.3.2. Transport of milk constituents

The macro and micronutrients of milk are secreted into the milk through different routes. In order for compounds to enter breast milk from the maternal circulation, first they must cross the polarized mammary epithelial barrier. There are five major pathways through which milk constituents are secreted into the milk (64). They are: Exocytosis (I), Transcytosis (II), Milk fat globule secretion (III), Transmembrane transporters (IV), and Paracellular route (V) (Figure 2.4.).

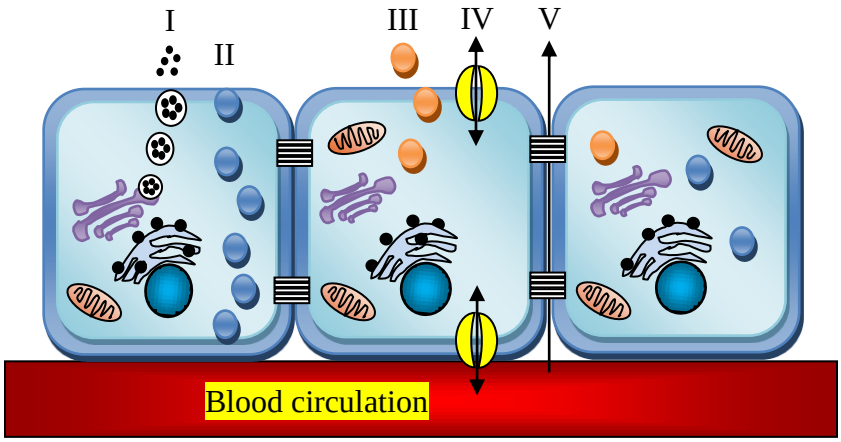

Figure 2.4. The major secretory pathways through which milk nutrients are secreted into the breast milk. I Exocytosis, II Transcytosis, III Milk fat globule, IV Transmembrane transporters, and V Paracellular route.

The major nutrients found in the aqueous phase of breast milk such as casein, whey proteins, lactose, and α-lactalbumin are secreted via the exocytosis route. The proteins that are secreted by this route are first synthesized on ribosomes within the mammary epithelial cells. They are then transported to the endoplasmic reticulum where they are folded, modified, and transported through the Golgi complex system. Subsequently, these proteins are secreted into the alveolar lumen by the exocytosis pathway (65, 66). Macromolecular substances or intact proteins such as immunoglobulins, transferrin, and prolactin enter into the milk within specialized vesicles through transcytotic processes (50, 64). Lipids, lipid associated proteins and lipid soluble hormones synthesized in the mammary gland epithelium are secreted via the milk fat globule route. In addition, various small molecules such as glucose, amino acids, sodium, and potassium are transported across the polarized mammary epithelium through substrate specific transporters (50, 66). The mechanism of transport varies with each compound that uses this route. For instance, glucose is transported by GLUT family of transporters through facilitated diffusion process, whereas Na^+ and K^+ concentration gradients are maintained by Na^+/ K^+ ATPase activity (64). During the lactation stage, the tight junctions between the adjacent mammary epithelial cells limit the paracellular transport of milk constituents (64). Hence, milk constituents are transported into the lumen of the mammary gland principally through transcellular routes (I-IV). However, these tight junctions become leaky during involution and

inflammatory conditions of mammary gland (i.e. mastitis), which leads to the free passage of milk constituents between the milk and the blood stream (67).

2.3.2.1. Transporters in the mammary epithelium

Hormonal regulation in pregnant and lactating mothers causes significant changes in the mammary gland including epithelial cellular proliferation and differentiation into polarized mammary epithelium, onset of milk production from mammary epithelial cells and significant changes in transporter expression in the mammary epithelium (68). Transporters play an important role in making the essential substrates available to mammary epithelial cells for cellular metabolic energy requirements, synthesis of milk constituents, and to provide critical macro- and micronutrients in the milk. The lactating mammary gland expresses a variety of transporters (69), which are classified into two main groups, the ATP-Binding Cassette transporters and the Solute Carrier Transporters.

2.3.2.1.1. ATP-Binding Cassette (ABC) transporters

ABC transporters are primary active transporters, which use ATP for the movement of substrate against or independent of a concentration gradient (70). These transporters are principally efflux transporters. They are expressed at the apical or basolateral surfaces of polarized epithelial barriers. Depending on their localization in a polarized epithelium these transporters can either accumulate compounds in a particular tissue or play a protective function by preventing the access of compounds into certain tissues (70).

2.3.2.1.2. Solute Carrier transporters (SLC)

SLC transporters are classified as facilitated diffusion transporters or secondary or tertiary active transporters, which do not directly use ATP as a driving force for the movement of substrates across the cellular membranes (71). These transporters either use the previously established concentration gradient for their substrate transport or they actively create a concentration gradient for the transport of the desired substrate. SLC transporters can be facilitators (movement of substrates across the cellular membranes based on an existing concentration gradients), co-transporters/symporters (simultaneous movement of a compound that creates energy-driving force along with the wanted substrate), and exchangers/antiporters (exchange of one substrate with another substrate) (71).

The expression of the transporters changes with the stage of lactation (64). With the onset of lactation, mammary gland epithelial cells have a high demand for precursor macronutrient molecules such as glucose and fatty acids to facilitate the production of major milk components, lactose and lipids in the breast milk. Glucose is transported into mammary epithelial cells through facilitative glucose transporters (GLUT) and sodium dependent secondary active Na^+/glucose transporter (SGLT) (50). Similarly, transport of fatty acids requires fatty acid binding proteins (FABP) and fatty acid transport proteins (FATP) (72). Apart from the glucose and fatty acids, other macronutrients such as amino acids and peptides are transported into mammary epithelial cells through various amino acid transporters and the oligopeptide (PEPT) transporters, respectively (73). Micronutrients such as zinc, iron, copper, vitamins, minerals, and hormones are transported into milk through specific transporter systems.

During lactation, metabolic activities of mammary epithelial cells also increase dramatically to meet the requirement for milk synthesis and secretion. Since glucose and fatty acids are the main energy substrates, the intermediate metabolites formed during its metabolism contribute significantly to the energy production in mammary epithelial cells (64, 74).

2.3.2.1.3. Monocarboxylate transporters

Monocarboxylates are formed in the metabolism of carbohydrates and lipids. The intermediate metabolites like lactate, pyruvate and short chain fatty acids contain 2-4 carbon atoms with a monocarboxyl group (COOH) in its structure and are known as monocarboxylates. These compounds provide fuels and materials for the use of and conversion into glucose and fatty acids in most cells. They require special transporter proteins to move across the cellular membranes and mitochondrial membranes in order to maintain cellular homeostasis and to produce more metabolic energy, respectively (75). For example, aerobic and anaerobic glycolysis of glucose gives pyruvate and lactate, respectively. Lactate reversibly converts into pyruvate in the presence of lactate dehydrogenase enzyme. Pyruvate needs a transporter to enter into mitochondria to enter the tricarboxylic acid cycle and electron transport chain process and to produce more cellular energy. Lactate produced during aerobic and anaerobic glycolysis, either needs to be converted to pyruvate or to be transported out from cells to maintain cellular homeostasis (75-77). Since lactose and fatty acids are the major nutrients for the nursing neonate, the MCTs in the mammary gland play an important role in making the lactate and short chain fatty acids available to the mammary epithelial cells and in production of energy (78).

Monocarboxylate transporters are classified into two types that are responsible for the uptake of monocarboxylates into epithelial cells (78). They are the proton-coupled monocarboxylate transporters (MCTs) and sodium-coupled monocarboxylate transporters (SMCTs).

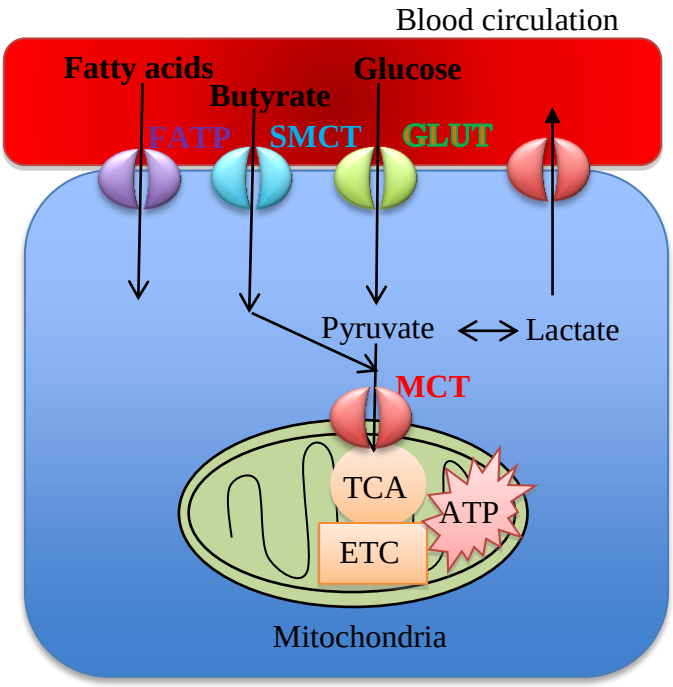

Figure 2.5. Uptake of energy substrates into mammary epithelial cells. Fatty acids, short chain fatty acids (butyrate) and glucose are transported into mammary epithelial cells through Fatty acid transporter (FATP), sodium dependent monocarboxylate transporter (SMCT), and glucose transporter (GLUT). The intermediate formed in the metabolism of glucose (pyruvate or lactate) is either transported out of the cell (lactate) or transported into to mitochondria (pyruvate) through monocarboxylate transporter (MCT) to enter the tricarboxylic acid cycle (TCA) followed by electron transport chain (ETC) to produce more cellular metabolic energy.

2.3.2.1.3.1. Proton-coupled MCTs

Proton-coupled monocarboxylic acid transporters belong to the SLC16A family of transporters. There are 14 members (MCT1 to 14) of this family of transporters described (79). So far, only 11 of these transporters have been identified as functionally active (MCT1 to 11). Of these 11, MCT1 distribution is ubiquitous, i.e. this transporter is present everywhere in the human body (78).

2.3.2.1.3.1.1. Monocarboxylate transporter-1

MCT1 functions as a proton dependent co-transporter or exchanger. MCT1 catalyses either the net transport of one monocarboxylate with one proton or the exchange of one carboxylate with another carboxylate (78). MCT1 plays a crucial role in the transport of monocarboxylates and ketone bodies like acetoacetic acid and β-hydroxybutyric acid across epithelial cell membranes and from cell cytosol to mitochondria for the production of cellular metabolic energy. Furthermore, MCT1 is involved in the uptake of pyruvate, lactate and other monocarboxylates for production of medium and long chain fatty acids (79). Since lactose and milk fat are the important constituents of milk, MCT1 may play an important role in synthesis of these constituents.

2.3.2.1.3.1.2. Sodium-coupled MCT

Sodium-coupled monocarboxylate transporters (SMCT) belong to the SLC5A family of transporters. Two types of SMCTs are present in the body – SMCT1 and SMCT2. SMCT function as Na^+-coupled electrogenic transporter for monocarboxylates, mainly short chain fatty acids (SCFAs). SMCTs are abundantly expressed in colon, liver, kidney, thyroid, uterus, ovaries, fallopian tubes and lactating mammary gland (80). In mammary gland, the mammary epithelial cells utilize SCFAs transported by SMCTs to produce milk fat and for cellular metabolic energy (79).

2.3.2.1.4. L-Carnitine transporters

In addition to these monocarboxylate transporters, carnitine transporters also transport intermediate metabolites of fatty acids. Carnitine is an important nutrient that has a number of essential roles in intermediate metabolism. It plays a crucial role in the transport of long-chain fatty acids across the inner mitochondrial membrane via carnitine acyltransferase enzyme system, so they can undergo β-oxidation to give acetyl CoA, which again produces usable cellular energy via the citric acid cycle (81, 82). Carnitine is also useful in removal of excess toxic fatty acyl-CoA metabolites and to maintain the balance between free and acyl-CoA. Carnitine is present either as free carnitine (FC) or as acylcarnitine (AC). A ratio of AC/FC is an index of mitochondrial function. A low ratio of AC/FC indicates a healthy mitochondria where as a high AC/FC indicates a decrease in mitochondrial function, which leads to decrease in energy production (81). In mammals, L-carnitine homeostasis is maintained by *de novo* synthesis of L-carnitine in liver and brain (25%) and by dietary intake (75%) (81, 83).

L-carnitine uptake into tissues is mediated through the organic cation/carnitine transporters (OCTNs), neutral and cationic amino acid transporter, $ATB^{0,+}$, and carnitine transporter 2 (CT2). Three types of OCTNs are expressed in mammalian tissues, namely, OCTN1, OCTN2 and OCTN3. OCTNs belong to the SLC22A family of transporters. OCTN1 and OCTN2 transporters show more than 70% similarity in their amino acid sequence, however, they show differences in their function (84).

OCTN1 is a sodium-independent organic cation transporter that transports cations in a bidirectional, pH dependent manner. It is a multispecific transporter and has low affinity for L-carnitine in species including rat and humans. It is abundantly expressed in intestine, liver, and kidney (85, 86). OCTN2 has high affinity for L-carnitine and transports L-carnitine in a sodium-dependent manner. The unique aspect of this transporter is its dual mode of transport: it transports organic cations via sodium independent process and L-carnitine via sodium dependent process. It is widely expressed in tissues such as heart, skeletal muscle, kidney, intestine, placenta, brain and mammary gland (87, 88). A mutation in the OCTN2 gene causes secondary L-carnitine deficiency (87). OCTN3 transports L-carnitine in a passive, sodium and pH independent manner. It has higher specificity for L-carnitine transport than OCTN1 and 2. It is predominantly expressed in testis (89, 90) and in peroxisomes (91).

Apart from OCTNs, ATB^{0+} also transports L-carnitine across cellular membranes. It belongs to the SLC6A family of transporters (92). The letter B of ATB^{0+} represents broad substrate specificity of the transporter system and superscript $^{0+}$ represents both neutral and cationic amino acids as substrates to this transporter. ATB^{0+} transports amino acids depending on Na+ and Cl- ions concentrations across the cell membrane. ATB^{0+} has low affinity for L-carnitine and is expressed more in the lung, colon, mammary gland, pituitary gland and ocular tissue. Furthermore, L-carnitine is also transported by L-carnitine transporter 2 (CT2). This novel transporter belongs to SLC6A family of transporters and is expressed in human testis (93). The localization of these transporters in tissues and at epithelial barriers is critical in understanding their endogenous role in cellular metabolism and function. For example, expression of OCTN3 in peroxisomal membranes plays a critical role in peroxisomal β-oxidation pathways (91). Expression of OCTN2 on the apical membrane on rat intestinal epithelium suggests that OCTN2 is responsible for uptake of L-carnitine into enterocytes.

To date, the majority of the literature discusses changes in expression of these transporters during lactation stage; however, very little information is known about the changes that occur to these nutrient transporters during inflammatory conditions of mammary gland. Since carbohydrates and lipids provide major nutrients in the milk, the mammary epithelial cells require special proteins to transport them into the milk. Inflammatory conditions alter the expression of these proteins at the mammary epithelium and, consequently, the composition of the milk may change during inflammatory conditions.

2.4. Inflammation and its effect on mammary epithelium

2.4.1. Effect of inflammation on epithelial barrier function

Exposure of pathogens to mucosal surfaces elicits epithelial responses that activate inflammatory processes (94, 95). A variety of bacteria and their secreted/excreted products show effects on epithelial barrier function and permeability by releasing pro-inflammatory cytokines. Release of these cytokines during bacterial infections may show its direct impact on the integrity of the epithelial barrier by increasing its permeability (96). For example, *in vitro* studies have shown that epithelial derived pro-inflammatory cytokines (TNF-α, IL-1β, and IFN-γ) have the potential to cause damage to epithelial cells through necrosis, apoptosis and loss of cell-to-cell contacts (97). Numerous studies have shown that TNF-α alters the barrier function in both epithelial and endothelial cells. For example, studies in polarized porcine renal epithelial cell monolayers, LLC-PK1, have shown that the TNF-α induced decrease in transepithelial electrical resistance (TEER) was followed by an increase in TEER, which exceeded control TEER by 300%. Furthermore, mannitol flux experiments confirmed the decrease in TEER was accompanied by increases in paracellular permeability, but the increase in TEER was not accompanied by a decrease in paracellular permeability of mannitol (98). TNF-α significantly reduced TEER and increased paracellular permeability of mannitol in HT29 cl.19A and HT-29/B6 cells (99, 100). Incubation of recombinant human IFN-γ with polarized T84 intestinal epithelial cells showed progressive decreases in TEER, which was accompanied by increase in mannitol flux in epithelial monolayer (101).

2.4.2. Epithelial cell metabolism and composition of milk

Changes in milk composition during inflammatory conditions have been postulated to occur as a result of impaired cellular synthesis, impaired cellular secretion, cellular degeneration

and impaired transport of milk constituents from blood to milk and from milk to blood (102). Inflammation of the mammary gland alters milk composition by affecting the synthetic and secretory activity of mammary epithelial cells. For example, bacterial toxins such as *E.coli* endotoxin and *Staphylococcus aureus* α-toxin inhibited the proliferation of bovine mammary epithelial cells (102). Endotoxin induced inflammatory response in mammary glands showed decreased concentrations of lactose in both cow and goat milk. This may be due to reduction in mammary epithelial cell secretory activity and to the movement of lactose from milk into the blood. Furthermore, mammary gland inflammation in cows and goats increased the concentration of sodium and chloride in milk (103, 104). The increased concentrations of these two ions in milk correlate significantly with decreased concentrations of milk lactose. Similarly, citrate concentrations were also decreased in endotoxin induced bovine mammary epithelial cell. In bovine milk, citrate is thought to be synthesized in or accumulated by the Golgi apparatus of the epithelial cells (105). Reduced concentrations of citrate in milk may indicate the impairment in mammary epithelial cell synthesis and secretion during endotoxin induced inflammation.

2.4.3. Effect of inflammation on nutrient transporters

Inflammation can change the expression, activity and function of many transporters at cellular membranes (74). *In vitro* studies in intestinal cell culture systems have shown that pro-inflammatory cytokine IFN-γ increased di/tri-peptide transporter expression (hPEPT1) (106). In primary hepatocytes, TNF-α and IL-6 decreased sodium-taurocholate co-transporting polypeptide (NTCP) and organic anion transporting polypeptide 1B1 protein expression and transport activities (OATP) (107). Intestinal inflammation down-regulates enterocytic MCT1 expression, which may lead to mucosal inflammation and Inflammatory Bowel Disease (108). Colonic tumorogenesis down-regulates SMCT1 expression in colon (80, 109). Expression of SMCT1 is downregulated in cancers of thyroid, breast, stomach, and pancreas (110-113).

Several *in vivo* studies have also identified changes in transporter expression during inflammatory conditions. For instance, intestinal inflammation decreased the expression and activity of intestinal fructose transporter (GLUT5) in various species. LPS challenged mice downregulated the renal tubular glucose transporters GLUT2, SGLT2 and SGLT3 with concomitant decrease in plasma glucose concentrations (114). These data suggest expression of transporters in the mammary gland may alter during inflammatory conditions. Previous research in our lab has shown that LPS induced inflammation in rat mammary gland altered the glucose

(Glut1, Glut8 and Sglt1), fatty acid (Fatp1, Fatp4 and Fabp3), and L-carnitine (Octn1, Octn2, and Octn3) transporters expression levels at different stages of lactation, day 4 and day 11. LPS caused a significant decrease in mRNA expression of transporters at each lactation stage except for Octn3 and Fatp1. The expression of Octn3 and Fatp1 was markedly increased with LPS administration at lactation day 4, and the expression of Sglt1 was slightly increased at day 11 of lactation (115). Furthermore, the pro-inflammatory cytokines are known to play a critical role in regulation of numerous protein expression during inflammation. Thus, it was postulated that changes in the expression of transporters probably occurred through the cytokine mediated pathways. If inflammation alters transporter expression levels, which, in turn, may affect the availability of substrates, cellular metabolism and thus the biosynthetic capacity of the mammary epithelium will be affected. To date, the majority of the studies have focused on LPS induced inflammation and its effects on the release of pro-inflammatory cytokines and on expression of transporters at epithelial barriers. Very few studies, though, have evaluated the influence of the Gram-positive bacterial stimulant, LTA, on the expression of transporters at epithelial barriers, especially the mammary epithelium. Previous unpublished studies in our laboratory have shown the differential changes in the expression of glucose, fatty acid and L-carnitine transporters during Gram-positive (LTA) and Gram-negative (LPS) bacterial inflammatory stimulation *in vitro* (74). Since the expression of glucose, fatty acid and L-carnitine transporters change with inflammation, mammary epithelial cells may need to make compensatory changes in energy substrate utilization to support cellular energy metabolism needs and cellular synthesis of milk constituents. Therefore, my research project will focus on the expression of Monocarboxylate and L-Carnitine (OCTN2-a high affinity carnitine transporter) transporters as energy substrate transporters.

2.5. Hypothesis

Inflammation of the mammary gland is a major concern in lactating women. Inflammation may create an unfavorable change in nutrient composition of milk by affecting the expression of nutrient transporters in the mammary gland. Changes in such transporter expression levels during mammary gland inflammation may affect the availability of energy substrates, their utilization, and the biosynthetic capacity of the mammary epithelium as well as epithelial barrier function in general. Since Gram negative (LPS) and Gram positive (LTA) bacteria are the most common pathogenic causes of inflammation in the mammary gland,

comparison studies will be performed using an *in vitro* human mammary epithelial cell line to know whether different bacterial inflammatory stimuli can cause different changes in the expression of transporters.

Hypothesis:

LPS and LTA differentially alter the mRNA and functional expression of energy substrate transporters in mammary epithelial cells.

2.5.1. Objective – I

To determine whether there are differential effects of LPS and LTA on mRNA expression of MCT1, SMCT1, and SMCT2 transporters in the normal immortalized human mammary epithelial cell line, MCF-12A, using real-time RT-PCR analysis.

2.5.2. Objective – II

To determine whether the changes in mRNA expression result in changes in cellular energy utilization and in transporter activity in MCF-12A cells. Specifically, this will involve:

- Measurement of high energy phosphate (AMP, ADP and ATP) and creatine compounds (creatine and creatine phosphate) using HPLC.
- An evaluation of whether changes in high energy phosphate and creatine compounds associate with changes in cellular oxygen levels, the cellular oxygen consumption rates were measured in LPS and LTA treated MCF-12A cells as compared with control.
- An evaluation of whether changes in mRNA expression of transporters cause changes in functional activity of transporters, uptake transport studies in MCF-12A cells were conducted using radiolabelled compounds.

3. MATERIALS AND METHODS

3.1. Materials

T-75 flasks, sterile 15 mL and 50 mL polypropylene centrifuge tubes, 12 well plates, 96 well plates, eppendorf tubes, bicinchoninic acid (BCA) protein assay kit, and 4 mL scintillation vials were purchased from Thermo Fisher Scientific (Ottawa, Ontario, Canada). Mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 medium (DMEM-F12) cell culture medium was purchased from American Type Culture Collection (ATCC) (Rockville, Maryland, USA). Quantitative reverse transcription-polymerase chain reaction (QRT-PCR) tubes and one-step SYBR green RT-PCR kits were acquired from Applied Biosystems (Foster City, California, USA). Ribonucleic acid (RNA) isolation midi kit was purchased from Qiagen Inc. (Toronto, Ontario, Canada). Enzyme-linked immunesorbent assay (ELISA) kits for interleukin-6 and interleukin-1β were purchased from R&D Systems, Inc. (Minneapolis, Minnesota, USA). Lipopolysaccharide (LPS from *Escherichia coli* strain O55:B5) and Lipotechoic acid (LTA from *Staphylococcus aureus*), Adenosine triphosphate (ATP), Adenosine diphosphate (ADP), Adenosine monophosphate (AMP), Creatine (Cr), Creatine Phosphate (CrP), L-carnitine, horse serum, trypsin, phosphate buffered saline, bovine insulin, epidermal growth factor and cholera toxin were purchased from Sigma-Aldrich (Toronto, Ontario, Canada). L-[methyl-^{3}H]Carnitine hydrochloride (83 Ci/mmol) and [^{14}C]-mannitol (58 mCi/mmol) were acquired from Perkin Elmer (Waltham, Massachusetts, USA). Ecolite was purchased from MP Biomedicals (Solon, Ohio, USA). High performance liquid chromatography (HPLC) grade methanol was purchased from Caledon Laboratories (Georgetown, Ontario, CA). Highly purified deionized water was obtained from a MilliQ Synthesis water purification system (Millipore, Bedford, MA). All other solvents and reagents used were of the highest analytical grade available.

3.2. LPS and LTA effects on mRNA expression of MCT1, SMCT1, and SMCT2 transporters in MCF-12A cells using QRT-PCR analysis

3.2.1. Cell culture

MCF-12A cell line, an immortalized non-cancerous human mammary epithelial cell line that has phenotypic characteristics of mammary epithelial cells *in vivo*, was purchased from ATCC at passage number 9. MCF-12A cells were cultured as specified by ATCC in a 1:1

mixture of DMEM and Ham's F-12 medium with 5% horse serum supplemented with 0.5 μg/mL hydrocortisone, 10 μg/mL bovine insulin, 0.1 μg/mL cholera toxin, and 20 ng/mL epidermal growth factor. The cells were maintained at 37°C under an atmosphere of 95% O_2 and 5% CO_2 in a humidified incubator. Cell culture media was changed every 2-3 days. At approximately 70-80% confluency cells were subcultured at a ratio of 1:2 to 1:4 using 0.25% trypsin. For all the experiments, MCF-12A cells were used at passage number 14.

3.2.2. LPS/LTA treatment

Previous optimization experiments in MCF-12A cells showed 1 μg/ml of Lipopolysaccharide (LPS) and Lipotechoic acid (LTA) induce maximal cytokine (TNF-α) expression without loss of cell viability (74). The stock solutions of LPS and LTA were prepared in water at a concentration of 2 mg/mL and 1 mg/mL, respectively, in 4 mL glass bottles. For LPS, the glass bottles coated with dichlorodimethylsilane (silanized) were used to prepare stock solutions and it was sonicated for 10-15 min before treating the cells. IL-6 and IL-1β ELISA kits were used to confirm the release of cytokines due to LPS and LTA stimulation of MCF-12A cells. Time course experiments were conducted to know the time-dependent changes in the mRNA expression of cytokines IL-6 and IL-1β.

MCF-12A cells were plated in triplicate on 12-well plates at a density of 3×10^4 cells/mL. 2 mL of the cell culture media along with cells were added to each well and incubated at 37°C for 48 h. After 48h, the cell culture media was replaced with fresh media containing 1 μg/mL of LPS or LTA and incubated for 6, 12, and 24 h. Cells not treated with LPS and LTA were considered as controls. At a given incubation time, the media was collected in 1.5 mL eppendorf tubes for ELISA analysis of IL-6 and IL-1β and stored at -80°C until analysis. ELISA for IL-6 and IL-1β were performed according to the manufacturer instructions using BSA as standard. The cells attached to the 12-well plate were washed twice with PBS and trypsinized using 200 μL of 0.25% trypsin/well. After a few minutes, 500 μL of cell culture media was added to each well in order to stop the action of trypsin. Cells along with the media were then collected in 1.5 mL eppendorf tubes and centrifuged at 100 rpm for 5-7 min. The media was aspirated out and the cell pellet was stored at -80°C for mRNA extraction followed by QRT-PCR. All experiments were repeated on three different occasions.

3.2.3. RNA isolation

Total RNA was extracted using RNeasy Midi Kits according to the manufacturer's instructions. Frozen cell pellets were thawed for 15-20 min at room temperature. Cell lysate was homogenized with 350 μL of buffer RLT containing β-ME. To this homogenate, 1 volume of 70% ethanol was added to precipitate the nucleic acids. The cell lysate was applied to the Midi column and series of buffers were added to remove the cellular contaminants according to the manufacturer's instructions. The purified RNA was isolated from the column using RNase-free water (30 μL).

The concentration and purity of the isolated RNA was determined by using Nanoview UV spectrophotometer (GE Healthcare Life Sciences, Quebec, Canada). Total RNA was quantified by measuring the absorbance of a diluted RNA (RNA:RNase-free water) at 260 nm according to the following formula:

$$\text{Concentration of RNA} = 40\ \mu g/ml \times A_{260} \times \text{Dilution factor}$$

RNA purity was assessed by measuring absorbance ratio (A_{260} / A_{280}) of a diluted sample of RNA (RNA:10 mM TrisCl (pH-7.5)). Pure RNA has a ratio between 1.9-2.1. Al1 samples used for QRT-PCR were of high purity. Total RNA was stored at -80°C until analysis.

3.2.4. Primer Design

Gene sequences for MCT1, SMCT1, SMCT2, IL-6, IL-1β and β-actin were obtained from the National Center for Biotechnology Information Gene Bank (NCBI) and specific primers were designed using Primer3 software (http://frodo.wi.mit.edu/). The forward and reverse sequences and amplicon size are given in Table 3.1.

Table 3.1. Primer details for mRNA expression analysis of transporters using QRT-PCR.

Gene	Accession number	Forward primer	Reverse primer	T_m (**ºC)**	Base pairs size
β-actin	NM_001101.3	ttgctatccaggctgtgc	atgtcacgcacgatttcc	86.1	235
IL-6	NM_0006000.3	acgaagctgcaggcacagaacc	atttgccgaagagccctcaggc	81.3	107
IL-1β	NM_000576.2	acagatgaagtgctccttccagga	gcccttgctgtagtggtggtcg	80.5	91
MCT1	NM_001166496	ccttttctgctcgtttgc	tggaatgctgtcctgtcc	81.6	189
SMCT1	NM_145913.3	cagcactcagcgtatttgg	gagttggcaaagggaacc	79.3	82
SMCT2	NM_178498.3	aacaggtcgccaaagagg	gcactgccattctcaagg	83.2	156

3.2.5. Quantitative Reverse transcription-polymerase chain reaction (QRT-PCR)

Quantitative RT-PCR is currently the "gold standard" for mRNA analysis offering the best sensitivity, dynamic range, and reproducibility of any standard technique. The relative expression of mammary epithelial monocarboxylate transporters and interleukins were determined using QRT-PCR. One step QRT-PCR reactions were performed using a SYBR Green RT-PCR reagent kit and an Applied Biosystems Real-Time PCR System. All reactions were performed in a final volume of 25 μL as per Table 3.2.

Table 3.2. Components of QRT-PCR master mix reaction. Adapted from Applied Biosystems (http://www.appliedbiosystems.com)

Component	Volume (μL)/Reaction	Final concentration
2X Quantitect SYBR green	12.5	1X
Quantitect RT mix	0.125	-
RNase inhibitor	0.5	-
Forward primer	2.0	<500 nM
Reverse primer	2.0	<500 nM
RNase free water	5.875	-
Template RNA	2.0	50 ng/μL
Total	**25**	**-**

Primers were optimized and validated for its most favorable annealing temperature and highest primer efficiency. The optimal annealing temperature produces a single melting peak for a specific product, which is not identified in a blank sample containing no RNA (a negative control). QRT-PCR products were further resolved by horizontal 2% (w/v) agarose gel-electrophoresis for a single band at the specified amplicon size correlating to the single, specific product melt peak, to assure a single PCR product as identified by a single band on the gel.

Primer efficiency was calculated from the slope of a 3-point standard curve and efficiencies of 1.8 to 2.2 were considered as optimal. Primers giving closer efficiency to the efficiency of β-actin (internal gene) were used for QRT-PCR reaction. The reactions were quantified following determination of the threshold cycle (C_T; the amplification cycle when PCR products are first detected above baseline fluorescence) and fluorescence was measured from the intercalation of SYBR green dye into the double stranded product after the primer elongation phase. A non-template negative control was incorporated into all analysis runs. PCR products were analyzed using comparative C_T or $2^{-\Delta\Delta C}{}_T$ method.

QRT-PCR reactions consisted of reverse transcription (1 cycle at 48ºC for 30 min) in order to make cDNA from RNA. Initial activation step (1 cycle at 95ºC for 15 min) was followed by a three step thermal cycling (40 cycles; denaturing at 94ºC for 15s, annealing at 60ºC for 30s, and extension at 60º for 30s). Finally, a melt curve analysis from 65ºC to 95ºC at 0.5ºC/s was performed.

3.3. Functional assessments in LPS and LTA treated MCF-12A cells

Cellular metabolism parameters such as creatine compounds (Creatine, Creatine phosphate), adenine nucleotides (ATP, ADP and AMP), oxygen consumption rates and uptake of radiolabelled L-carnitine were measured to determine whether changes in transporter expression due to LPS and LTA induced inflammation in MCF-12A cells associate with altered cellular energy metabolism, cellular oxygen consumption rates, and uptake of cellular nutrients, respectively.

3.3.1. Measurement of high-energy phosphates (ATP, ADP and AMP) and creatine compounds (Cr and Cr P)

High energy phosphate substrates (ATP, ADP and AMP) and creatine compounds (creatine (Cr) and creatine phosphate (Cr P)) were measured using HPLC-UV as described by Olkowski et al. (116). The HPLC (Agilent Technologies, Mississauga, ON) system consisted of a series 1200 quaternary pump with an online degasser, autosampler and diode array detector. Processed samples (10 µL) were injected onto a C18 column (Pursuit XRs 5C18 250×3.0 mm). The analytes were eluted under gradient conditions (Table 3.3.) at a flow rate of 0.7 mL/min and absorbance was monitored at 210 nm. The mobile phase was filtered through a 0.22 µm Nylon filter (Pall Scientific, Mississauga, ON) and degassed in an ultrasonic bath for 30 min prior to

use. The column was maintained at 20°C and washed with water:methanol (50:50) after every use.

Table 3.3. Gradient assay of HPLC-UV method to measure Cr, Cr P, ATP, ADP and AMP levels in MCF 12A cells.

Time (min)	20mM Phosphate buffer (pH-5.5)	Methanol	Water	Flow rate (mL/min)
0.0	100	0	0	0.7
17.0	100	0	0	0.7
17.5	0	95.0	5.0	0.4
19.5	0	95.0	5.0	0.4
20.0	0	5.0	95.0	0.4
22.0	0	5.0	95.0	0.4
23.0	100	0	0	0.5
28.0	100	0	0	0.7

Total run time for each sample was 38.5 min

3.3.1.1. Standards preparation

Primary stock solutions for Cr, CrP, ATP, ADP and AMP were prepared at a concentration of 1 mg/mL by dissolving in phosphate buffer (pH-5.5). From this stock solution, 10 µg/mL of Cr, CrP, ATP and ADP, 20 µg/mL of AMP were prepared and diluted serially 2 fold times to get a standard curve for 6 different concentrations (10 to 0.312 µg/mL for Cr, CrP, ATP, & ADP, and 20 to 0.625 µg/mL for AMP). High quality control (HQC), mid quality control (MQC) and low quality control (LQC) samples at 8, 4, and 0.5 µg/mL for Cr, Cr P, ATP, ADP and 16, 8 and 1.5 µg/mL for ATP were prepared using the stock solution independent of those concentrations used for the standard curve. Primary stock solutions were stored at -20°C.

3.3.1.2. Sample preparation

MCF12A cells were plated in 12-well plates. After reaching 70-80% confluency, cells were treated with 1 μg/mL of LPS and LTA for 12 h. After 12 h, cell culture media was removed and cells were washed twice with phosphate buffered saline (PBS). 0.35 mL of 0.7 M perchloric acid was added to each well and a cell scraper was used to detach the cells from the surface of the well. The cell lysate was homogenized and centrifuged at 12,000 rpm in an Eppendorf microcentrifuge (Accuspin Micro 17, Fisher Scientific) for 7 min. 100 μL of the supernatant was collected in a new eppendorf tube, neutralized with 0.25M KOH to bring the pH to 7.0 and then filtered through 0.45 μm filter (Pall life sciences, Mississauga, ON). 10 μL of this filtrate was injected onto the HPLC analytical column. Concentration of the high energy phosphates and creatine compounds was calculated by interpolation from the linear calibration curve ($r^2 > 0.98$) using external standards. Intra- and interday accuracy and precision was within 15%.

3.3.2. Measurement of oxygen consumption rate

MCF-12A cells grown in T-75 flask were treated with 1 μg/mL of LPS or LTA. After 12 h, cell viability was assessed with Trypan blue exclusion method. Cells were re-suspended in 5 mL of freshly prepared cell culture media at a density of 5×10^6 cells/mL. The cell suspension was placed on water bath at 37°C during the experiment. Oxygen uptake by MCF-12A cells was measured with a Clark-type oxygen electrode at 37°C using Hansatech Oxytherm Measurement System (Hansatech, Norfolk, UK). The oxygen electrode was stabilized for at least 30 min with 1 mL air-saturated deionized water in the chamber. The water was replaced with 1 mL of cell suspension and the baseline was measured for 5 min. After every use, the chamber was washed with 70% alcohol followed by 1.5 mL of de-ionized water. Oxygen consumption rates were obtained by calculating the slope of the linear plot of oxygen (nmole/ml) vs time (min), and the consumption rates were measured as nmole/10^6 cells/min. Cell free media was used as negative control.

3.3.3. Uptake of radiolabelled L-carnitine in MCF-12A cells

MCF-12A cells were seeded at a density of 2×10^4 cells/well in a 96-well plate. Plates were incubated under an atmosphere of 95% O_2 and 5% CO_2 for 48 h. Optimization experiments were conducted to know the time dependent uptake of 10 nM [^{3}H]-L-carnitine and 0.86 μM [^{14}C]-Mannitol for 120 min in order to determine the incubation time period for concentration dependent studies. Mannitol was used as an extracellular marker for membrane integrity.

Concentration dependent uptake studies were performed using 10 nM [^{3}H]-L-carnitine and 0.86 μM [^{14}C]-Mannitol in the presence of 0.01 μM to 100 μM of unlabeled L-carnitine to determine the kinetic parameters (Vmax and Km) for a specific transporter system.

Previous studies in our lab have shown the temporal changes in mRNA expression levels of OCTN2 transporter due to LPS and LTA stimulation in MCF-12A cells. In continuation of those previous studies, I conducted experiments related to functional outcomes of OCTN2 transporter. For this, temporal changes in OCTN2 transport function due to different bacterial stimuli were determined by treating MCF-12A cells with 1μg/mL of LPS and LTA for 12 h and 24 h. After specific time period, the cell culture media was replaced with OCTN2 uptake buffer (25 mM Tris/HEPES, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, pH 7.5). Cells were preincubated at 37°C and 4°C for 20 min. Following the pre-incubation, the kinetic parameters of cellular uptake of L-carnitine by hOCTN2 transporter was studied by adding 100 μL of OCTN2 buffer containing 0.01μM [^{3}H]-L-carnitine and 0.86 μM [^{14}C]-mannitol in the presence of 0.01 μM to 100 μM of unlabeled L-carnitine. The cells were incubated for 1 h at 37°C and 4°C for both total and nonspecific uptake of substrates. The nonspecific uptake (4°C) values were subtracted from the total uptake (37°C) values to obtain the specific uptake of L-carnitine by carnitine transporters. After the incubation time had expired, uptake buffer was removed and cells were washed twice with 200 μL of ice-cold PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8 mM Na_2HPO_4) in order to stop the uptake of L-carnitine. Subsequently, 100 μL of 0.01N NaOH was added to each well and incubated at 37°C for 2 h to solubilize the cells. 25 μL aliquots of each sample were added to 4 mL of Ecolite liquid scintillation cocktail. The radioactivity was measured after 15 h using a Beckman LS 6500 Scintillation Counter (Beckman Coulter Inc., Mississauga, ON). All experiments were performed in triplicate on three separate occasions. Protein concentration was measured using BCA protein assay kit and BSA as the standard (Thermo Fisher Scientific, Canada).

3.4. Statistical Analysis

QRT-PCR data were analyzed using two-way ANOVA analysis followed by Tukey's multiple comparison test. High energy phosphates compounds, creatine compounds, and oxygen consumption rates were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. A P-value of 0.05 or less was taken to indicate a significant difference between the means of sets of data. All data were expressed as mean ± standard error of mean (SEM).

3.4.1. Estimation of transporter kinetic parameters

The kinetic parameters for L-carnitine uptake were estimated from total and nonspecific initial uptake rates at various substrate concentrations. Specific uptake of L-carnitine was obtained by subtracting the nonspecific uptake from total uptake. To estimate kinetic parameters of specific transport, the uptake rates were fitted to the following equations (two-site specific binding) by means of nonlinear least-squares regression analysis using GRAPHPAD PRISM 5 (San Diego, California, USA) program.

$$\vartheta = \frac{VmaxHi \times [C]}{KmHi + [C]} + \frac{VmaxLo \times [C]}{KmLo + [C]}$$

Where $VmaxHi$, $VmaxLo$ and $KmHi, KmLo$ denotes the maximum transport rate and half-saturation concentration for high and low affinity transporter systems, respectively. ϑ and [C] denotes the uptake rate and concentration of the substrate (L-carnitine), respectively. All data were expressed as mean ± SEM. The data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test, to compare the means of three sets of data. Each experiment was performed in triplicate, giving nine samples at each time point per LPS or LTA treatment. Significance level was set at $P<0.05$.

4. RESULTS

4.1. LPS and LTA differentially alter the mRNA expression of monocarboxylate transporters and interleukins in human mammary epithelial cells

To determine whether gram-negative and gram-positive inflammatory stimuli can differentially elicit an innate immune response, MCF12A cells were exposed to LPS and LTA for 6, 12, 24 h and cytokine mRNA and protein expression levels were evaluated. Previous research in our lab has shown the time-dependent differential changes in mRNA expression of TNF-α at 6 and 12 h in LPS and LTA treated MCF-12A cells (74). In continuation of that research, the cells were treated with LPS and LTA for different time periods to determine the time-dependent changes in the mRNA expression of monocarboxylate transporters (MCT1, SMCT1 and SMCT2) and interleukins (IL-6 and IL-1β) (Figure 4.1and Table 4.1). LTA and LPS caused no significant changes in IL-6 expression in MCF-12A cells (Table 4.1). LPS initially decreases IL-1 β mRNA expression at 6 h, but returned to control levels at 12 and 24 h (Table 4.1). LTA increased IL-1 β mRNA expression at 6h and 24h in MCF-12A cells.

LPS and LTA exposure altered the mRNA expression of MCT transporters in a time-dependent manner and the extent and pattern of change varied depending upon the inflammatory stimulus (Table 4.1). LTA increased MCT1 expression 3-fold after 6 h exposure. However, by 12 and 24 h post exposure MCT1 expression was less than 50% of control levels in MCF-12A cells. MCT1 expression was reduced by almost 50% relative to control following 24 h exposure to LPS. LTA exposure had no effect on SMCT1 mRNA expression, while expression levels were reduced almost 60% at 6h LPS exposure, with recovery to control levels by 12 and 24 h. LTA decreased SMCT2 mRNA expression relative to control at all time points, with expression at 10% control levels at 6h of exposure. LPS increased SMCT2 mRNA levels over 3-fold relative to control but levels were 60% of control by 24 h exposure.

Cell culture supernatants following 6 or 12 h exposure to 1 μg/mL LPS or LTA were used to determine the protein concentrations of interleukin (IL-6 and IL-1β) using ELISA kits. LPS increased IL-6 protein levels at 6 and 12 h but not IL-1β (Table 4.2). LTA had no effect on IL-6 protein concentrations, but decreased IL-1β levels at 12 h exposure when compared to control (Table 4.2).

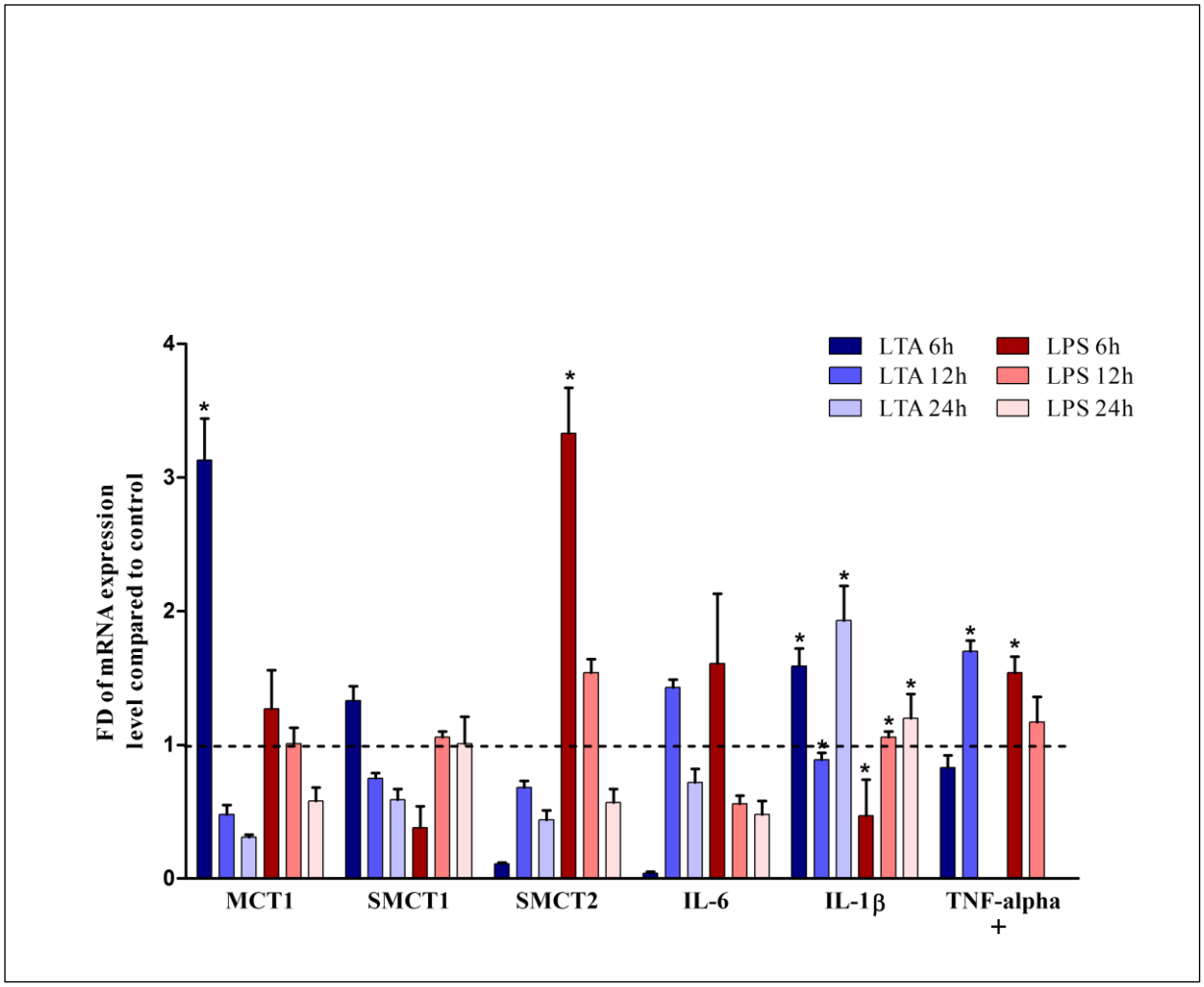

Figure 4.1. Mean ± SEM of fold diffenences (FD) in mRNA expression of monocarboxylate transporters (MCT1, SMCT1, & SMCT2), interleukins (IL-6 & IL-1β) and tumor necrosis alpha (TNF-α). mRNA expression of various transporters in MCF-12A cells incubated with 1 µg/mL of LPS/LTA for 6, 12, and 24 h was normalized to β-actin and fold difference (FD) was determined by using $2^{-\Delta\Delta CT}$ method. FD of means were compared using two-way ANOVA ($P < 0.05$) followed by Tukey's test to know the interaction between treatment (LPS and LTA) and time.

Bars with (*) showed significant interaction between treatment (LPS and LTA) and time

+ Previously reported mRNA expression of TNF-α

Table 4.1. Mean ± SEM fold differences in mRNA expression levels of various transporters and interleukins in MCF-12A cells incubated with 1 µg/mL of LPS/LTA for 6 h, 12 h and 24 h (n=3).

Target gene	LTA			LPS			P-Value
	6 h	12 h	24 h	6 h	12 h	24 h	Compared to Control
MCT1	$3.13 \pm 0.31^{*,a}$	0.48 ± 0.07^{b}	0.31 ± 0.02^{b}	1.27 ± 0.29^{a}	1.01 ± 0.12	0.58 ± 0.10^{b}	1.20
SMCT1	1.33 ± 0.11	0.75 ± 0.04^{b}	0.59 ± 0.08^{b}	0.38 ± 0.16	1.06 ± 0.04^{b}	1.01 ± 0.20^{b}	1.19
SMCT2	0.11 ± 0.01^{a}	0.68 ± 0.05^{b}	$0.44 \pm 0.07^{b,c}$	$3.33 \pm 0.34^{*,a}$	1.54 ± 0.10^{b}	$0.57 \pm 0.10^{b,c}$	1.63
IL-6	0.04 ± 0.01^{a}	1.43 ± 0.06^{b}	0.72 ± 0.10^{b}	1.61 ± 0.52^{a}	0.56 ± 0.06	0.48 ± 0.10^{b}	1.10
IL-1β	$1.59 \pm 0.13^{*,a}$	$0.89 \pm 0.05^{*,a,,b}$	$1.93 \pm 0.26^{*,a,c}$	$0.47 \pm 0.27^{*,a}$	$1.06 \pm 0.04^{*,a}$	$1.20 \pm 0.18^{*,a,b}$	0.00017

mRNA expression was normalized to β-actin and fold differences were determined using comparative C_T method.
Multiple comparisons for fold difference caused by LPS and LTA with different incubation times for each target gene were analyzed using two-way ANOVA with Tukey's Multiple Comparison test. Significance level was set at $P<0.05$ and noted for interaction effects.
Means with (*) showed significant interaction between treatment (LPS and LTA) and time
Within the treatment differenced were analyzed by using One-way ANOVA with Tukey's Multiple Comparison test ($P<0.05$).
Means with (^{a}) were significantly different between the treatments.
Means with (^{b,c}) were significantly different when compared to 6 h and 12 h of exposure within the treatment, respectively.

Table 4.2. Mean ± SEM of interleukin concentrations (pg/mL) in MCF-12A cells treated with 1 µg/mL of LPS and LTA for different time periods.

Interleukins	Control (pg/mL)		LTA (pg/mL)		LPS (pg/mL)	
	6 h	12 h	6 h	12h	6 h	12 h
IL-6	139.0 ± 5.71	143.25 ± 5.85	146.1 ± 8.32[b]	147.7 ± 6.03[b]	217.8 ± 6.76 [a,b]	185.44 ± 14.4[a,b]
IL-1β	99.2 ± 22.5	145.47 ± 12.8	129.4 ± 19.2	81.2 ± 14.69[a]	101.8 ± 13.4	102.46 ± 5.23

Interleukin concentrations of cells treated with LPS and LTA for 6 h and 12 h were determined by using ELISA kits. Data was analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Significance level was set at $P<0.05$.
Means with ([a]) were significantly different from control.
Means with ([b]) were significantly different from other treatment.

4.2. LPS and LTA differentially alter the cellular function of MCF-12A cells

4.2.1. Creatine and high energy phosphate substrate levels and oxygen consumption rates in LPS and LTA treated MCF-12A cells

To determine whether changes in the transporter mRNA expression levels due to LPS and LTA alter high energy substrate levels and oxygen consumption rates in MCF-12A cells, the concentration of high energy substrates (Cr, CrP, ATP, ADP and AMP) and oxygen consumption rates (OCR) were measured in MCF12A cells after 12 h of exposure with LPS or LTA. Figure 4.2 represents the HPLC chromatograms of blank (mobile phase) (A) and the standards of creatine compounds (Cr and CrP) and high energy phosphate substrates (ATP, ADP and AMP) spiked in mobile phase (B). Table 4.3 represents the changes in the creatine compounds and high energy phosphate substrate concentrations due to LPS and LTA exposure in MCF-12A cells. LPS significantly increased the concentration of Cr, CrP, ATP and ADP, whereas LTA caused significant changes only in CrP and ADP concentration levels as compared to control. AMP concentrations were not detected in the LPS and LTA treated MCF-12A cells.

To determine whether changes in high energy phosphates and creatine compounds can be due to changes in cellular oxygen levels, the oxygen consumption rates in LPS and LTA treated MCF-12A cells were compared with control cells. Figure 4.3. indicates a 28% and 42% decrease in oxygen consumption rates (OCR) in LPS and LTA treated MCF 12A cells, respectively, when compared to control. Furthermore, LTA decreased OCR 14% further than LPS in MCF-12A cells.

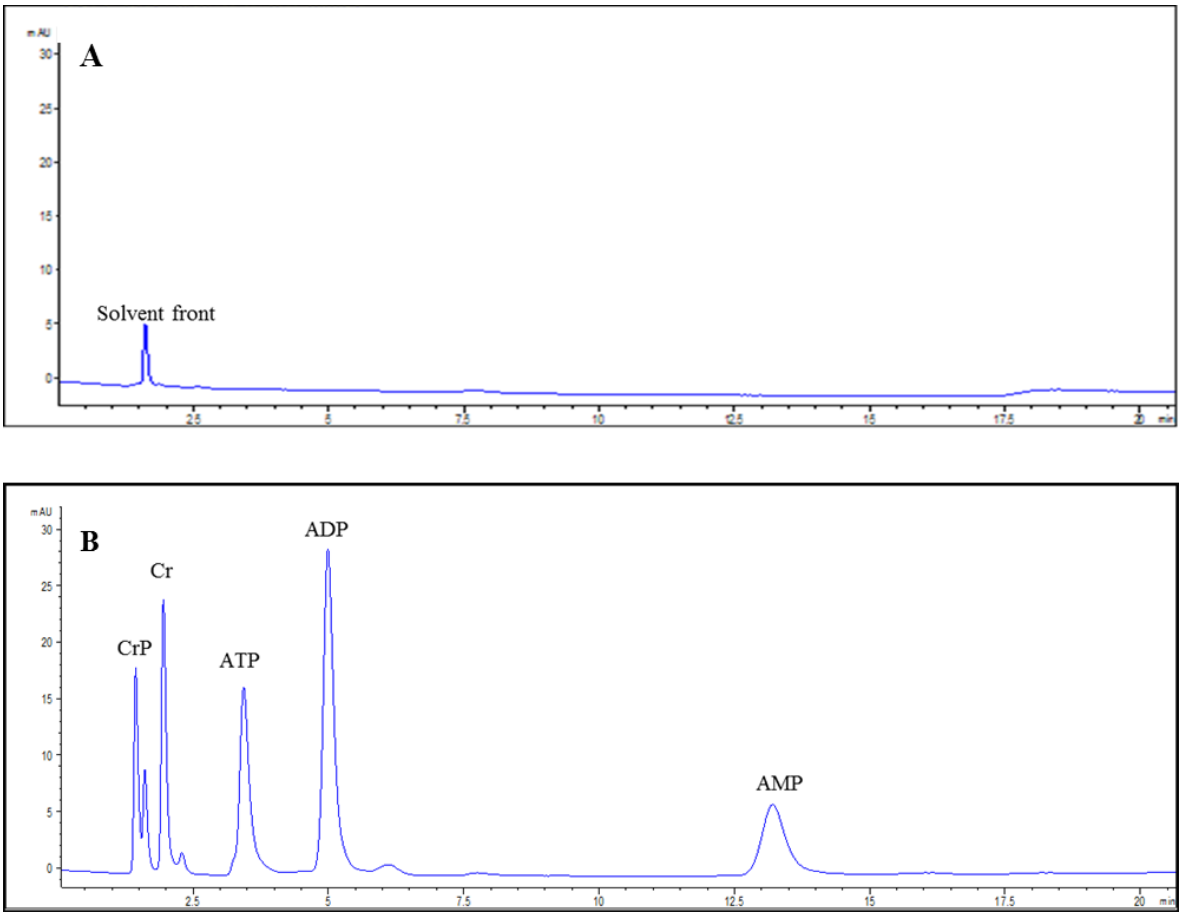

Figure 4.2. HPLC chromatograms of mobile phase (phosphate buffer, pH-5.5) (A), and phosphate buffer spiked with CrP, Cr, ATP, ADP (10 µg/mL) and AMP (20 µg/mL) (B).

Table 4.3. Mean ± SEM concentrations of ATP, ADP, Cr P and Cr (µg/mL) in MCF-12A cells incubated with 1 µg/mL of LPS or LTA and cell culture media (control) for 12 h.

High energy and Creatine phosphates	Control	LTA	LPS
ATP	2.04 ± 0.06	2.12 ± 0.09	2.43 ± 0.08*
ADP	0.42 ± 0.01	0.46 ± 0.03*	0.48 ± 0.02*
CrP	3.54 ± 0.03	4.22 ± 0.02*	3.98 ± 0.03*
Cr	0.31 ± 0.01	0.30 ± 0.01	0.32 ± 0.01*

Data was analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Significance level was set at $P<0.05$.
Means with (*) were significantly different from control.

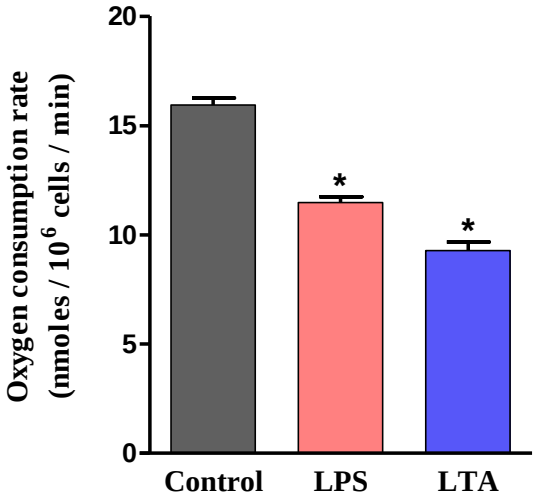

Figure 4.3. Mean ± SEM oxygen consumption rate in MCF-12A cells incubated with cell culture media (control) and 1 µg/mL of LPS or LTA for 12 hours. Means (n=3) were compared using one-way ANOVA ($P < 0.05$) followed by Tukey's multiple comparison test.

4.3. Active uptake of L-carnitine in MCF-12A cells

4.3.1. Time course of [^{3}H] L-Carnitine uptake by MCF 12A cells

The time course of 10 nM [^{3}H] L-carnitine by MCF-12A cells was determined by incubating the cells with radiolabelled OCTN2 uptake buffer (pH 7.5) for various time periods. As shown in Figure 4.4., MCF-12A cells accumulated [^{3}H] L-carnitine in a time dependent way and the uptake was linear up to 60 min. On the basis of these results, in subsequent experiments the cells were exposed to 10 nM of [^{3}H] L-carnitine for 60 min, to measure the initial uptake rate.

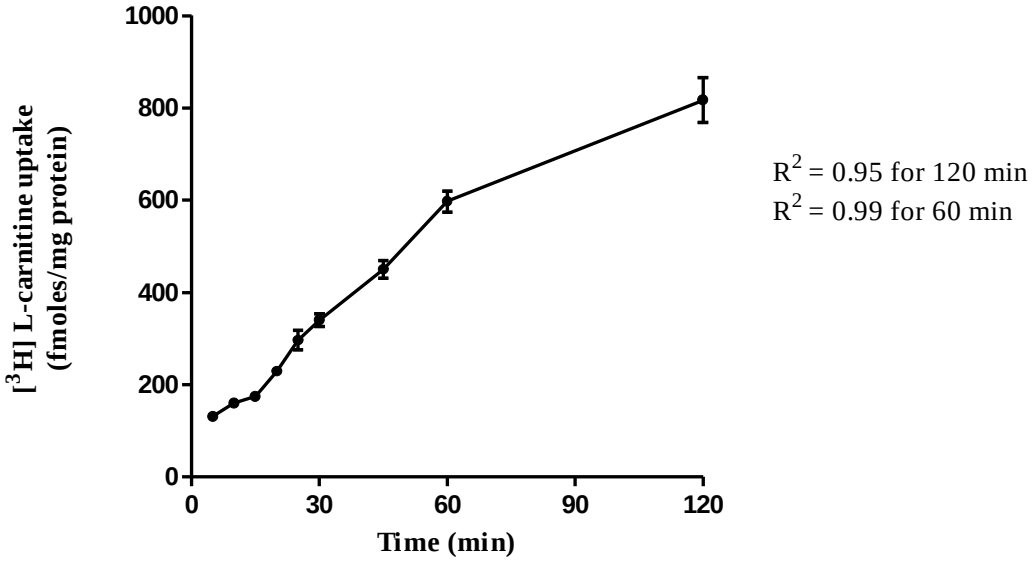

Figure 4.4. Time course of 10 nM [^{3}H] L-carnitine uptake by MCF-12A cells was measured at various time points by incubating in OCTN2 buffer (pH-7.5, n=3) at 37ºC. Uptake was linear up to 60 min. The results are shown as Mean ± SEM (n=3).

4.3.2. Concentration dependence of [^{3}H] L-carnitine uptake by MCF-12A cells

Previous studies in our lab have shown the time differential changes in mRNA expression levels of OCTN2 transporter due to LPS and LTA stimulation in MCF-12A cells. In continuation of those previous studies, the carrier-mediated transport of L-carnitine in MCF-12A cells was characterized by performing concentration dependent uptake studies. The concentration dependence of L-carnitine uptake in MCF-12A cells was investigated at 37ºC and 4ºC for total and nonspecific uptake, respectively. Specific uptake of L-carnitine was obtained by subtracting

nonspecific uptake from total uptake. The uptake of [^{3}H]L-carnitine was measured at concentrations between 0.01 and 100 µM in order to determine the kinetic parameters (Figure 4.5.) and data was fitted to equation 1 (shown in Materials and Methods). The kinetic parameters of the high affinity transport system are as follows: K_m = 3.01 ± 1.07 µM, V_{max} = 192.45 ± 5.71 pmoles/mg protein/hr.

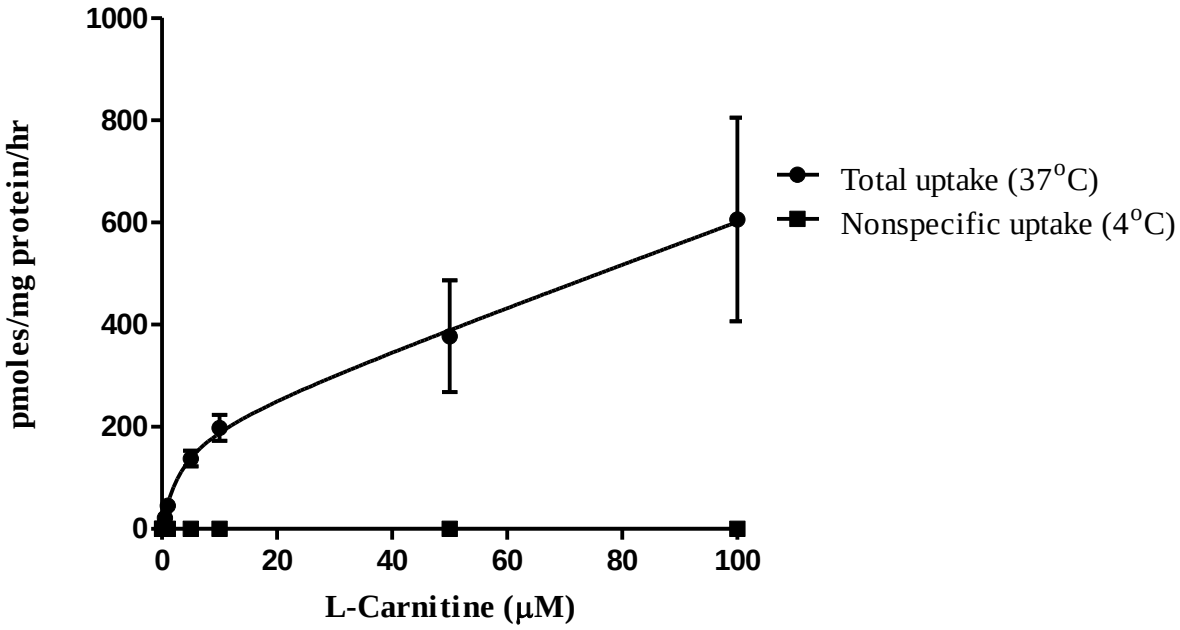

Figure 4.5. Concentration dependence of saturable uptake of 10 nM of [^{3}H]L-carnitine in human MCF-12A. Uptake of L-carnitine by MCF-12A cells was measured for 60 min in transport buffer (pH-7.5) at 37ºC and 4ºC in the presence of 0.01 and 100 µM unlabeled L-carnitine for total and nonspecific uptake, respectively. Kinetic parameters for specific uptake were determined by subtracting nonspecific uptake from total uptake of L-carnitine. Each result represents the mean ± SEM (n=3).

4.3.3. OCTN2 mediated uptake of L-carnitine in LPS and LTA treated MCF-12A cells

To determine whether LPS and LTA alters the kinetic parameters of carrier mediated L-carnitine transport, MCF-12A cells were treated with LPS and LTA. Figure 4.6. represents the specific uptake of L-carnitine in MCF-12A cells incubated with LPS and LTA for 12 h and 24 h. The kinetic parameters of control, LPS and LTA treated cells at different time periods are given in Table 4.4

Table 4.4. Kinetic parameters (V_{max} -pmoles/mg protein/hr, K_m -µM) of L-carnitine transport in MCF-12A cells treated with 1 µg/mL of LPS and LTA for 12 h and 24 h.

Kinetic parameters	Control		LTA		LPS	
	12 h	24 h	12 h	24 h	12 h	24 h
V_{max}	194.7 ± 6.54	190.2 ± 4.88	383.7 ± 23.23	172.7 ± 6.35	468.3 ± 22.8	224.7 ± 7.82
K_m	3.3 ± 1.3	2.7 ± 0.85	4.4 ± 0.68	2.1 ± 0.9	5.3 ± 0.69	3.8 ± 0.23

Mean ± SEM (n=3) of kinetic parameters of L-carnitine transport in LPS or LTA treated MCF-12A cells were analyzed using One-way ANOVA analysis. There is no significant difference between Control, LPS and LTA treated cells.

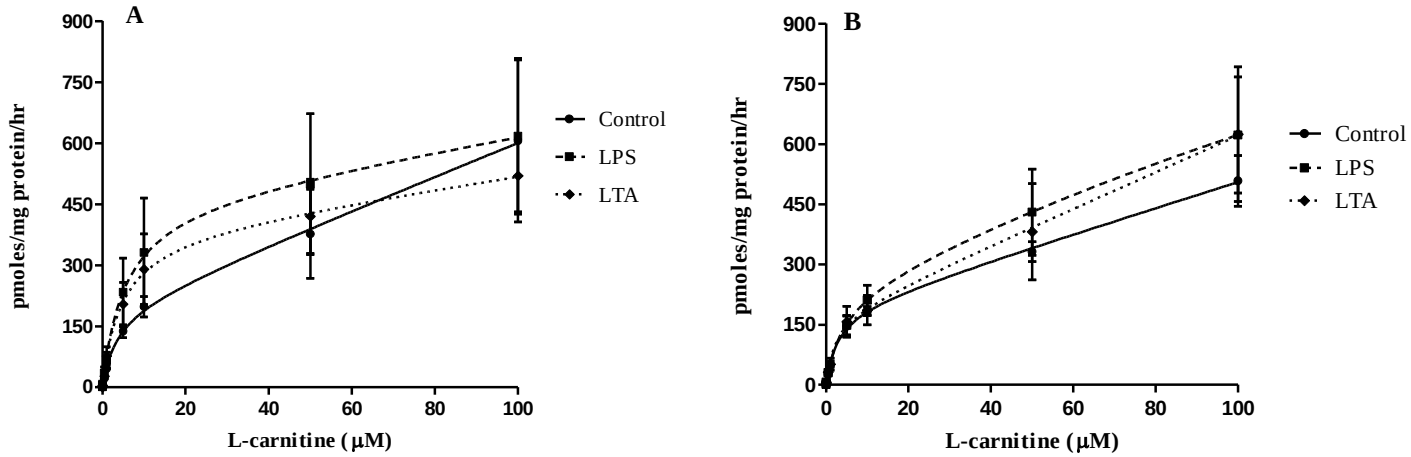

Figure 4. 6. Concentration dependent uptake of 10 nM [^{3}H]L-carnitine in MCF-12A cells treated with LPS and LTA. Cells grown in 96-well plates were treated with 1 µg/mL of LPS and LTA for 12 h (A) and 24 h (B). Uptake of carnitine by MCF-12A cells was measured for 60 min in transport buffer (pH-7.5) at 37ºC and 4ºC in the presence of 0.01 and 100 µM unlabeled L-carnitine for total and nonspecific uptake, respectively. Kinetic parameters for specific uptake were determined by subtracting nonspecific uptake from total uptake of L-carnitine. Each result represents the mean ± SEM (n=3).

Since I noted no statistically significant changes in L-carnitine transport activity in LPS and LTA treated MCF-12A cells, I did not evaluate the kinetic parameters for glucose, monocarboxylate, and fatty acid transporter systems as these assays require very short transport times (and, therefore, are technically very difficult to conduct).

5. DISCUSSION

Pathogenesis of mastitis due to LPS and LTA may involve different inflammatory stimuli to alter transporter gene expression and, therefore, substrate availability in human mammary epithelial cells. In my study, comparison of the expression of monocarboxylate transporter-1, sodium coupled monocarboxylate transporter-1, sodium coupled monocarboxylate transporter-2 and cytokines (TNF-α, IL-6 and IL-1β) between these two types of bacterial challenges has been carried out in the normal human mammary epithelial cell line, MCF-12A. The monocarboxylate transporters make critical substrates available to mammary epithelial cells that support epithelial cellular energy requirements (117). My data suggest LPS and LTA inflammatory stimuli differentially alter the mRNA expression of proton-coupled and sodium-coupled monocarboxylate transporters and cellular metabolism in MCF-12A cells. In addition to this, exposure of LPS and LTA in mammary epithelial cells showed qualitative and quantitative differences in the time course of the innate immune response (TNF-α, IL-6 and IL-1β) and in the mRNA expression of transporters.

According to previous reports, LPS from *E. coli* and LTA from *S. aureus* are widely used as model bacterial challenges in mammalian cells to induce cytokine release (118, 119). Furthermore, I have used MCF-12A cell line for my studies, which is an immortalized human mammary epithelial cell line known to have similar phenotypic characteristics of mammary epithelial cells *in vivo* (120), and has been used in previous investigations as a cell culture model to examine the effects of LPS and cytokine challenge on epithelial cell function and its gene expression (13, 121, 122). Mammary epithelial cells act as a primary interface between pathogens and the internal milieu and are a crucial site for innate immune responses. Considering these characteristics, optimization experiments were conducted with mammary epithelial cells to determine the appropriate concentrations of LPS and LTA to induce maximum cytokine expression without loss of cell viability.

LPS showed concentration dependent cytotoxicity, but not LTA (13). Both LPS and LTA induced time-dependent changes (6 and 12 h) in expression of TNF-α mRNA in MCF-12A cells. LPS induced the highest TNF-α expression at earlier time points (1 and 6 h), whereas LTA induced a more delayed increase in TNF-α expression (Figure 4.1.). These observations concurs with data reported in primary bovine mammary epithelial cells where LTA was less potent than

LPS in stimulating TNF-α in mammary epithelial cells (13). In primary bovine mammary epithelial cells, LPS and LTA caused rapid stimulation of TNF-α in the first 2-4 h but returned to control levels by 8-16 h following LTA challenge, while LPS showed a sustained response in TNF-α expression (13). Furthermore, Lahouassa et. al. also showed rapid induction of TNF-α in LPS and LTA treated bovine mammary epithelial cells followed by a decrease in its expression at 10 h (123). However, studies in the bovine mammary gland showed that LTA (*S. aureus*) induced infection elevated the transcriptional activity of TNF-α up to 24 h followed by sharp decrease at 32 h (124). Species differences, differences in cell source (primary versus immortalized) and cellular environment (*in vitro* versus *in vivo*), and differences in the concentrations of LPS and LTA used to challenge mammary epithelial cells likely explain these differences in TNF-α expression in LPS and LTA induced cells.

Similar to the TNF-α expression, LPS caused rapid induction of IL-6 mRNA at early time points (6 h), while LTA caused maximum induction at 12 h and returned to control levels by 24 h (Figure 4.1.). Differences in the induction of TNF-α and IL-6 mRNA by LPS and LTA in human mammary epithelial cells may suggest that MCF-12A cells have the ability to respond differentially to bacterial challenges. Furthermore, delayed induction of the pro-inflammatory cytokines, TNF-α and IL-6, may explain, in part, why LTA causes chronic inflammation compared to acute inflammation induced by LPS. The lack of immediate response towards LTA is not clear. Though, I have noticed differences in the intensity of IL-1β responses in LTA challenged cells, the IL-1β levels were initially decreased and then returned to base level at 12h and 24 h in LPS challenged cells. *E. coli* challenged infection in bovine mammary gland showed increase in IL-1β at 16 h post infection, whereas *S. aureus*-induced infection showed peak levels of IL-1β at 32 h post infection (124). Differences in experimental design (*in vivo* versus *in vitro*) again may explain the temporal differences in expression of IL-1β. More interestingly, at protein levels IL-6 and IL-1β differences were even more marked. In MCF-12A cells LTA does not cause any significant change in IL-6 protein, whereas LPS increased IL-6 protein levels and did not alter the IL-1β protein levels (Table 4.2.). Differences in mRNA and protein levels were also observed in cytokine expression of IL-6, IL-10 and TNF-α in horses with bronchial inflammation (125, 126). The lack of association between the mRNA of cytokines to its protein levels was not unexpected because regulation of protein levels is a complex process. Protein levels in the cytoplasm is not only governed by the amount of mRNA and the rate of translation, but also by

its folding efficiency, biochemical modification, degradation, and its transport in/from the cytoplasm (126).

LPS and LTA influenced the nature of the innate immune response by causing temporal differences in the induction of pro-inflammatory cytokines in mammary epithelial cells, which compelled me to investigate further whether these two different bacterial stimuli might lead to differences in the mRNA expression of monocarboxylate transporters. Pro-inflammatory cytokines may have differential or overlapping effects on expression of such transporters. Although LPS and LTA induce pro-inflammatory cytokines, they could regulate transporter expression through additional mechanisms that do not involve cytokines and autocrine regulation.

Transporters are responsible for the movement of energy substrates or milk nutrients or milk precursors across the mammary epithelial cell membranes. Expression and function of these transporters are known to change during inflammatory conditions (114). Previous unpublished studies in my lab have shown the differential changes in expression of glucose, fatty acid and L-carnitine transporters in LPS and LTA challenged mammary epithelial cells (74). Short-term exposure (6h) of LPS caused increased expression of glucose transporters (GLUT1, GLUT8, SGLT1), while LTA decreased glucose transporter expression and increased fatty acid transporters (FATP1, FATP4, FABP3) expression upon long-term exposure (12 h). Prolonged exposure of LPS increased the expression of the L-carnitine transporter, OCTN2, and fatty acid transporters, FATP1 and FATP4 (74). Limited information is available on monocarboxylate transporters and sodium-coupled monocarboxylate transporters in mammary epithelium during inflammatory conditions. Therefore, in my study I investigated whether the two different bacterial stimuli (LPS and LTA) might lead to differential changes in the mRNA expression of MCT-1 and SMCT1 and SMCT2.

Both LPS and LTA altered MCT1, SMCT1, and SMCT2 transporter expression in MCF-12A cells. With regard to the qualitative and quantitative differences in transporter expression levels, LPS and LTA caused significant differential effects on MCT1 and SMCT2 transporter expression, where LPS caused significant upregulation of SMCT2 transporter and LTA caused upregulation of MCT1 at 6 h followed by gradual decrease in its expression with LTA at 24 h (Figure 4.1.). My observations related to MCT1 expression are consistent with Thibault R et. al., who reported that TNF-α treatment in HT-29 cells showed a concentration-dependent

downregulation of MCT1 mRNA expression (108). However, Hahn et. al., reported that TNF-α upregulates both MCT1 mRNA and protein levels in macrophages (127). Further investigation is needed to know the role of TNF-α in regulating MCT1 expression.

LTA caused significant upregulation of MCT1 transporter at 6h and upon chronic exposure it caused down regulation of MCT1. Expression of MCT1 is identified both on the cellular plasma membrane (128) and at the mitochondrial membrane (129). This transporter is responsible for transport of pyruvate, lactate, and butyrate across the epithelial and mitochondrial membranes for their metabolism to produce ATP via oxidative phosphorylation. In rat and human colonocytes, butyrate provides the major source of ATP through fatty-acid β-oxidation and tricarboxylic acid cycle in mitochondria (130, 131). Butyrate oxidation on normal human and rat colonic epithelial cells produced more ATP than glucose, acetate, and propionate oxidation (132). Changes in MCT1 transporter expression may lead to a metabolic switch (butyrate to glucose) (133), which was shown in HT-29 colonic cells where butyrate oxidation decreased through downregulation of MCT1 expression and was associated with increased expression of GLUT1 transporter and glucose oxidation (108). Interestingly, MCT1 expression is significantly downregulated in colonic epithelial cells during its transition from normal to malignant phenotype and this transition is associated with increased expression of GLUT1 transporter (108). Furthermore, when compared with previous unpublished work in my lab, the upregulation of MCT1 with short-term exposure to LTA was accompanied by a downregulation of glucose transporters.

Several studies on intestinal epithelial cells have identified that inflammation downregulates the expression of MCT1 and SMCT1 (134, 135). Although, I did not find statistically significant differences in SMCT1 expression in both LPS and LTA challenged cells relative to control, prolonged incubation of LTA caused a gradual decrease in the expression of MCT1 and SMCT1 transporters while SMCT1 expression recovered with increased time of LPS exposure [138]. Interestingly, the expression of SMCT1 is silenced in a variety of cancers suggesting that this transporter is associated with a tumor suppressive function (135, 136). The significant role of SMCT1 to transport butyrate across epithelial cells provides an important clue to the mechanism involved in several carcinomas. Butyrate inhibits histone deacetylase enzyme, which plays a key role in determining cellular gene expression, and has been shown to cause cellular growth arrest and apoptosis in a variety of tumors (137). Furthermore, Bingham et. al.,

have shown the evidence for lower levels of butyrate in colon and the development of colorectal cancer (138). Reduction in expression of MCT1 and SMCT1 transporters upon prolonged LTA exposure suggest that development of chronic inflammation associated with gram-positive bacterial challenge may associate with silencing of these transporters and loss of the beneficial effects of butyrate. The recovery of SMCT1 expression with prolonged exposure to a gram negative bacterial stimulus may explain in part why mammary epithelial cells recover more quickly from gram negative bacterial infections as the ability to restore butyrate transport allows butyrate to mediate its beneficial effects on cellular gene expression and energy production.

Regarding SMCT2, very limited information is available about its expression during inflammatory conditions. Depending on its location in the proximal tubule of the kidney and proximal part of the intestine, SMCT2 may play a critical role in lactate transport in epithelial cells (139, 140). LPS challenge caused a marked increase in SMCT2 expression while LTA decreased SMCT2 expression at 6 h of exposure in MCF-12A cells. The ability to transport lactate from the cell is critical to prevent cellular acidosis and subsequent damage as a result of intracellular lactate accumulation. How the differential response in SMCT2 expression following LPS and LTA challenge influences mammary epithelial cellular function is uncertain but may warrant further investigation.

Since LPS and LTA causes differential changes in transporters that mediate the transport of critical substrates used in the generation of cellular energy, I next evaluated whether such changes could result in differences in mitochondrial ATP production. LPS significantly increased ATP, ADP, CrP, and Cr levels while oxygen consumption rate decreased, whereas LTA increased ADP and CrP and decreased the oxygen consumption rate in mammary epithelial cells. Increased expression of SMCT2 and decreasing utilization of oxygen in LPS treated cells may suggest that they are generating their ATP through aerobic and/or anerobic glycolysis and the enhanced lactate production is addressed via the SMCT2 transport function. The LPS induced increased in GLUT expression as shown previously in my lab also supports the idea of enhanced glycolytic flux as increase glucose uptake into the cell is possible with enhanced GLUT expression. An enhanced glycolytic flux with reduction in oxygen consumption rate (and thus oxidative phosphorylation) may explain the increase in ATP levels in LPS challenged mammary epithelial cells. Furthermore, the increased levels of CrP and ADP in LPS and LTA treated cells also helps to explain the maintenance of cellular ATP levels. ATP/CrP ratio in LPS and LTA

treated cells (0.6 and 0.5) is consistent with physiological ATP/CrP ratio of the myocardium (0.5-0.8). However, the oxygen consumption rate in LTA treated cells was significantly reduced when compared to control, yet ATP levels were unchanged. Further studies are needed to know why LTA caused decrease in oxygen consumption rate.

At last, I have performed uptake studies for specific transporter using a radiolabelled substrate. Considering the previous work in my lab, changes in mRNA expression of L-carnitine directly correlates with L-carnitine milk levels in the lactating mammary gland and, specifically, reduced expression of Octn2 transporter correlated well with reduced milk L-carnitine concentrations (141, 142). L-Carnitne transporter uptake studies were performed using [^{3}H]L-carnitine in order to determine whether LPS/LTA induced changes in the mRNA expression of transporter (74) leads to changes in substrate availability. Mammary epithelial cells express several carnitine transporters such as ATB^{0+} (143), OCTN1 (86, 144), and OCTN2 (145). Since, OCTN2 has high affinity to transport L-carnitine, uptake studies for OCTN2 transporter were conducted. Exposure of LPS and LTA to mammary epithelial cells did not alter the affinity (K_m) of OCTN2 transporter to transport L-carnitine. My estimate of K_m value (3.01 μM) (Figure 4.5.) is consistent with reported values of hOCTN2 (3.5 to 5.1 μM) in various expression systems (145, 146). In addition to this, the K_m values in MCF-12A cells challenged with LPS and LTA for 12h and 24h (2.1 to 5.3 μM) were also consistent with the reported values. Although, I noted no statistically significant changes in V_{max} of the OCTN2 transporter between control and LPS/LTA challenged cells, at 12 h the V_{max} was increased more than twice in LPS treated cells and close to twice in LTA treated cells when compared to control (Table 4.4.). The increase in V_{max} corresponds to the differential increase in mRNA expression of OCTN2 transporter in LPS and LTA treated MCF-12A cells, observed previously in my lab (74). Hence, LPS and LTA induced changes in mRNA expression of transporters may cause changes in the functional activity of the transporters.

6. SUMMARY OF FINDINGS

The principle aim of this thesis is to know whether gram-positive and gram-negative bacteria can differentially alter the expression of energy substrate transporters in human mammary epithelial cells. LPS and LTA challenge to the human mammary epithelial cell line, MCF-12A, showed differences in the induction of pro-inflammatory cytokines, which might suggest that both these bacteria induce inflammation in mammary epithelial cells differentially. Corresponding to the different bacterial inflammations, MCF-12A cells showed qualitative and quantitative differences in the time course and mRNA expression of transporters MCT1, SMCT1 and SMCT2. Although it is not significant in all transporters, prolonged exposure of LTA caused reduction in MCT1, SMCT1, and SMCT2 transporter expression, suggesting that gram-positive bacterial inflammation may associate with down-regulation of these transporters and to the development of chronic inflammation in the mammary gland. However, the return of expression to control or slightly decreased levels upon long term exposure with LPS may explain the quick recovery of mammary epithelial cells during gram-negative bacterial infections.

Functional assessments in LPS and LTA treated MCF-12A cells showed differences in creatine, high energy phosphate levels and cellular oxygen consumption rates. LPS exposure to mammary epithelial cells significantly increased ATP, ADP, Cr, and CrP levels despite a decrease in oxygen consumption rates. Increased expression of SMCT2 and decreased usage of oxygen in LPS challenged cells may suggest that these cells generate ATP by aerobic and/or anerobic glycolysis. LTA exposure to mammary epithelial cells decreased the oxygen consumption rate and increased ADP and CrP levels without changing ATP levels. Further investigations are needed to know why oxygen utilization is significantly decreased in LTA treated cells.

LPS and LTA exposure to mammary epithelial cells did not alter the affinity (K_m) for carnitine transport. Although not statistically significant, LPS and LTA challenge increased the V_{max} of carnitine transport 12 h post LPS/LTA exposure to MCF-12A cells when compared to control. The increase in V_{max} values correlate with the differential changes in the mRNA expression of the high affinity carnitine transporter, OCTN2, observed previously in my lab. Together, these data suggest that LPS and LTA induced changes in mRNA expression of

transporters might cause changes in the functional activity of the transporters and, in turn, to unfavourable changes to overall function of mammary epithelial cells during bacterial infection.

7. FUTURE DIRECTIONS

- *In vitro* studies showed the mRNA expression of MCT1, SMCT1, and SMCT2 transporters in MCF-12A cells. Further studies will be needed to determine protein expression and localization on polarized epithelial cellular membranes.
- Expression of MCT1 on the cellular membrane requires an ancillary protein CD147, or basigin. Further investigation will be needed to determine CD147 expression during a bacterial challenge, how it may change during inflammatory conditions, and its effect on activity of MCT1 transporter.
- Toll-like receptors (TLRs) play a key role in recognizing the different bacterial cell wall components. Studies will be required to know the expression of these TLRs in MCF-12A cells, and on the polarized epithelial cell membrane.
- Since, *in vitro* studies do not represent the complexity of the lactating mammary gland *in vivo*, such as contribution of immune cells to the inflammatory response, substrate availability through blood circulation, and differences in expression of transporters with the stage of lactation, *in vivo* investigations are necessary to complement the proposed *in vitro* studies.
- The individual role of pro-inflammatory cytokines in LPS and LTA induced inflammation and its role in modulating the transporter expression at different stages of lactation will be known by performing *in vivo* studies.
- *In vivo* studies will be required to evaluate the changes in mRNA expression of transporters in LPS and LTA induced inflammation could lead to changes in the nutrient composition at different stages of lactation and how it will affect the nursing neonates growth and development.

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